Role of ChemR23 Signaling in Inflammation

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Summary

ChemR23 is a G-protein coupled receptor expressed on monocytes and macrophages as well as on adipocytes. It binds two ligands, the peptide chemerin and the omega-3 fatty acid derived resolvin E1 (RvE1). Chemerin is present in inflamed tissue and acts as a chemoattractant of ChemR23 expressing leukocytes. Chemerin is also an adipokine secreted by mature adipocytes and regulating preadipocyte maturation. The second ChemR23 ligand RvE1 induces resolution of inflammation. It has been shown protective in many animal models of chronic and acute inflammation and is believed to be one of the mediators of the beneficial properties of dietary omega-3 fatty acids. As a receptor of chemerin and RvE1, ChemR23 plays a role in macrophage immigration to the inflamed areas, in inflammation resolution as well as in adipogenesis and might present a connection between inflammation and adiposity. Yet, little is known about the regulation of ChemR23 during different stages of inflammation or in different macrophage phenotypes.

Our results show that ChemR23 is predominantly expressed in the inflammatory M1 macrophages, which are attracted via the chemerin-ChemR23 axis. In contrast, the anti-inflammatory M2 macrophages do not express the receptor and are not responsive to ChemR23 mediated signaling. The proresolving action of RvE1 must therefore be on inflammatory M1 or naive macrophages. Our data show that RvE1 treatment of M1 macrophages increased transcription of the anti-inflammatory cytokines IL-10 but does not cause full re-polarization into M2 phenotype. We further show that ChemR23 is transcribed from 2 transcription starts in monocytes and macrophages and that the usage of promoters upstream of these starts differs in naive and classically activated cells. As a results, several ChemR23 mRNA isoforms differing in the 5´untranslated region are transcribed in monocytes and naive and activated macrophages likely enabling phenotype specific regulation of mRNA translation.

Plasma levels of the ChemR23 ligand chemerin have been associated with obesity, chronic inflammation and metabolic syndrome - risk factors for coronary artery disease (CAD). We studied possible direct association of chemerin levels with CAD. Our data show that in CAD patients not taking low dose aspirin, plasma chemerin levels are significantly higher than in patients on low dose aspirin and in healthy controls. On cellular level, we show that inflammatory cytokines typically secreted by inflammatory M1 macrophages increase chemerin production in adipocytes. Low dose aspirin treatment has no direct impact on chemerin production in adipocytes *in vitro* but decreases inflammatory cytokine secretion in

M1 macrophages thus decreasing the stimulus for chemerin production in adipocytes. Our data indicate that chemerin levels might reflect the inflammatory status of adipose tissue and that even low dose aspirin has anti-inflammatory effect on M1 macrophages.

CAD is caused by the formation of atherosclerotic plaques in the aterial intima, in which inflammation plays an important role. Polymorphisms in the ChemR23 gene may have an impact on inflammation and thus on the onset and/or course of CAD. We sequenced the coding region of ChemR23 gene and of the two promoters used in monocytes and macrophages in a CAD case-control study and analyzed the associtaion of idenfied polymorphisms with CAD. Our results suggest an associtaion of the promoter polymorphism rs1399820 and a trend for associtation of the non-synonymous polymorphism rs192034694 in the coding region with CAD. While our data indicate, that rs192034694 might have an impact on ChemR23 functionality, a functional assay would have to confirm this notion.

Zusammenfassung

ChemR23 ist ein G-Protein gekoppelter Rezeptor, welcher von Monozyten und Makrophagen wie auch von Adipozyten exprimiert wird. ChemR23 bindet zwei Liganden: das Peptid Chemerin und das von den Omega-3-Fettsäuren abstammende Resolvin E1 (RvE1).

Chemerin ist in entzündetem Gewebe vorhanden und verhält sich als Chemoattraktant für Leukozyten, die ChemR23 exprimieren. Zusätzlich ist Chemerin ein Adipokin, welches von reifen Adipozyten sezerniert wird und die Reifung der Präadipozyten reguliert. Der zweite ChemR23 Ligand, RvE1, veranlasst die Resolution der Entzündungsreaktion. RvE1 hatte eine schützende Wirkung in verschiedenen Tiermodellen der chronischen und akuten Entzündung und es wird vermutet, dass es einer der Vermittler der nutzbringenden Eigenschaften der Omega-3-Fettsäuren ist.

Als Chemerin und RvE1 Rezeptor spielt ChemR23 eine wichtige Rolle bei der Migration von Makrophagen in das Entzündete Gewebe, bei der Resolution der Entzündung, wie auch bei der Adipozytenreifung. Allerdings ist bis jetzt die ChemR23 Regulation während den verschiedenen Entzündungsstadien wie auch in den unterschiedlichen Makrophagen Phänotypen relativ unbekannt.

Unsere Ergebnisse zeigen, dass ChemR23 überwiegend in den pro-inflammatorischen M1 Makrophagen exprimiert wird, welche über die Chemerin-ChemR23 Achse angezogen werden. Im Gegensatz dazu exprimieren die anti-inflammatorische M2 Makrophagen kein ChemR23 und können somit nicht auf das Chemerin oder RvE1 Signal eingehen. Die proresolutive Aktion von RvE1 kann daher nur auf M1 oder auf unaktivierte Makrophagen wirken. Unsere Daten weisen darauf hin, dass in RvE1 behandelten M1 Makrophagen die Transkriptionsrate des anti-inflammatorisches Zytokines IL-10 erhöht ist ohne dass eine volle Repolarizierung in M2 Phänotyp stattfindet. Zusätzlich deuten wir darauf hin, dass ChemR23 von zwei unterschiedlichen Transkriptionsstartpunkten in Monozyten und Makrophagen transkribiert wird. Die Nutzung der Promotoren unterscheidet sich zwischen den naiven und M1 aktivierten Zellen. Somit werden unterschiedlich lange Isoformen von ChemR23 mRNA erhalten, die sich in der 5´nicht-transkribierten Region unterscheiden. Vermutlich ermöglicht diese Diversifikation eine Phänotyp spezifische Regulation der Transkription und Translation.

Die Chemerin Plasmakonzentration wurde mit verschiedenen Risikofaktoren für die koronare Herzkrankheit verknüpft, namentlich Adipositas, chronische Entzündung und metabolisches Syndrom. Wir haben untersucht, ob die Chemerin Plasmakonzentration direkt mit der koronaren Herzkrankheit verbunden ist. Wir zeigen, dass Chemerinkonzentration in Patienten

mit koronare Herzkrankheit, die kein niedrigdosiertes Aspirin zu sich nehmen, signifikant erhöht ist. Auf zellulärer Ebene zeigen wir, dass Inflammatorische Zytokine, die typischerweise von M1 Makrophagen sezerniert werden, die Chemerinproduktion in den Adipozyten erhöhen. Behandlung mit niedrigdosiertem Aspirin hat keine direkte Auswirkung auf die Chemerinproduktion in den Adipozyten *in vitro*, erniedrigt allerdings die Produktion der inflammatorischen Zytokine in den M1 Makrophagen. Folglich sinkt dann der Stimulus für die Chemerinproduktion in den Adipozyten. Unsere Werte weisen darauf hin, dass die Chemerinkonzentration den Entzündungsstatus des Fettgewebes widerspiegelt und dass selbst niedrigdosiertes Aspirin ein Anti-Entzündungseffekt auf M1 Makrophagen hat.

Entstehung und Entwicklung von atherosklerotischen Plaques, die koronaren Herzkrankheiten verursachen, ist verbunden mit dem Entzündungsprozess in der Plaque. Polymorphismen im ChemR23 Gen könnten einen Einfluss auf den Entzündungsprozess und somit auf den Krankheitsverlauf von koronaren Herzkrankheiten haben. Wir haben die Protein kodierende Region von ChemR23, wie auch die in Monozyten und Makrophagen aktiven Promotoren in einer Fall-Kontrolle Studie für koronare Herzkrankheiten sequenziert und untersucht of identifizierte Polymorphismen mit der koronaren Herzkrankheit verbunden sind. Der Promotorpolymorphismus rs1399820 zeigt einen deutlichen Zusammenhang mit koronaren Herzkrankheiten, wohingegen der funktionelle Polymorphismus rs192034694 nur eine Tendenz hat. Funktionelle Studien müssen durchgeführt werden, um den Einfluss der Polymorphismen auf die ChemR23 Transkription und Funktionalität zu zeigen.

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List of abbreviations

AA – amino acid

APC - antigen presenting cell

BMI – body mass index

CAD - coronary artery disease

COX - cyclooxygenase

CRP - C-reactive protein

DCs – dendritic cells

DHA - docosahexanoic acid

EPA – eicosapentanoeic acid

ER – endoplasmatic reticulum

GPCR – G-protein coupled receptor

HDL – high density lipoprotein

LDL – low density lipoprotein

LOX – lipoxygenase

LPS – lipopolysacharide

LTB_4 – leukotriene B_4

LXA_4 – lipoxin A_4

MMP – matrix metaloproteinase

NET - neutrophil extracellular trap

NK cells – natural killer cells

PGE_2 – prostaglandin E_2

PPARγ - peroxisome proliferator-activated receptor gamma

PUFA - polyunsaturated fatty acid

ROS – reactive oxygen species

RvD1 - resolvin D1

RvE1 – resolvin E1

T2D - type 2 diabetes

TLR - toll-like receptor

TXA – thromboxane A

UTR – untransladed region

WHO – world health organization

WT – wild type

1 Introduction

1.1 Inflammation

Inflammation is an essential protective response of the body to pathogen infection or tissue injury with the aim to remove the pathogen and/or damaged cells, and to return the tissue to homeostasis. Start of inflammation is marked with heat, redness, swelling and pain caused by increased blood flow, increased blood vessel permeability and inflammatory mediators sensitizing the local neural system in the affected area [1]. If inflammation is not resolved timely it can lead to tissue fibrosis and loss of function [2]. Impaired resolution of inflammation can furthermore lead to chronic inflammation, which is a key component of many diseases such as arthritis, inflammatory bowel disease and atherosclerosis [3].

1.1.1 Cellular basis of inflammation

Inflammation is initiated when microbial particles or fragments of own damaged cells are recognized by the tissue resident antigen presenting cells (APCs) such as macrophages or dendritic cells [4]. They start rapidly secreting signaling molecules to attract further leukocytes from the blood stream. Neutrophils are the first leukocytes recruited to the site of inflammation [5]. They are responsible for killing and phagocytosis of the pathogens. Activated neutrophils secrete reactive oxygen species (ROS), reactive nitrogen species, and other cytotoxic molecules to kill pathogens and detoxify the cellular debris [6]. In addition, neutrophils can extrude so called neutrophil extracellular traps (NETs), which are composed of DNA and histones and proteins from neutrophil granules. NETs have recently been shown to trap microorganisms and promote the interaction of these microorganisms with the neutrophil granule derived proteins and their destruction [7].

At the same time, mediators released from neutrophil granules attract monocytes from the circulation and activate surrounding endothelial cells to express adhesion molecules and chemoattractants facilitating monocyte extravasation [8]. Neutrophils are short-lived cells and phagocytosis of microbial particles or components of necrotic cells further induces their apoptosis [9]. Extravasation of monocytes to the site of inflammation is followed by their differentiation into macrophages, the main scavengers of apoptotic neutrophils [10]. Timely clearance of dead neutrophils is essential as cytotoxic substances contained within the neutrophils could lead to damage of the own tissue [11]. Phagocytosis of apoptotic neutrophils by macrophages leads to secretion of signals stopping further recruitment of

neutrophils and promoting the repair of adjacent tissue [2]. Finally macrophages are cleared by emigration into draining lymphatics [12].

1.1.2 Macrophage subsets

Macrophages play an important role in all stages of inflammation. They participate in its initiation by recognizing the pathogen and acting as APCs [4]. Subsequently they help detoxify and remove the pathogen. Finally, they play a key role in the resolution of inflammation and return of the affected tissue to homeostasis by removing apoptotic neutrophils and releasing molecules signaling for tissue repair [8]. To be able to fulfill all these functions, macrophages are very heterogeneous. They develop different phenotypes depending on the signals they receive from the environment. Several macrophage phenotypes with distinct functions have been described - the classically activated inflammatory M1 macrophages and alternatively activated M2 macrophages [13,14]. In addition, each tissue harbors resident macrophages, which were named according to their location – microglia (brain), kupfer cells (liver), alveolar macrophages (lung) [15].

M1 macrophages are important during the initiation of inflammation. They are activated by toll-like receptor (TLR) ligands such as the lipopolysacharide (LPS) a component of bacterial cell wall, and by T helper 1 (Th1) cell cytokines such as interferon-γ (IFN-γ) [4]. M1 macrophages secrete high amounts of inflammatory cytokines (IL-1β, TNFα) and ROS and remove pathogens by phagocytosis. They also further promote Th1 responses [14].

In contrast, M2 macrophages have regulatory and anti-inflammatory functions with a role in resolution of inflammation and tissue repair. M2 macrophages are considered to be cells activated by IL-4 or IL-13 expressing high levels of the mannose receptor and secreting the anti-inflammatory cytokines IL-10 and TGFβ [16]. However, there is a range of M2-like phenotypes, which are adopted by macrophages in response to IL-10, TGFβ or glucocorticoids [14]. These cells share some but not all characteristics with M2 macrophages. It is not clear whether M2 macrophages differentiate from newly arrived monocytes during the end of inflammation, whether they are created by proliferation of resident macrophages upon stimulation by Th2 cell cytokine [17], or whether M1 macrophages switch their phenotype to M2. It has however been shown that macrophage polarization state is not definite and that macrophages are at least partially able to switch from one phenotype to another *in vitro* as well as *in vivo* [18,19].

1.1.3 Chemokine and chemoattractant signaling

The above described interplay of lymphocytes during the course of inflammation is coordinated by a complex network of cytokine and chemokine signaling. Tight regulation of chemokine and cytokine release and of their receptor expression on different cell types allows extreme fine-tuning of the inflammatory response.

Chemotactic cytokines or chemokines are peptides, which are secreted by lymphocytes, epithelial and endothelial cells at specific time points of inflammation. Their activity can be further modified by cleavage by proteases such as metalloproteinases released from neutrophile granules [8]. Chemokines signal through G-protein coupled receptors (GPCRs) containing 7 membrane-spanning domains, differentially expressed on leukocytes, endothelial and epithelial cells. Chemokines are key in orchestrating the immigration of different types of leukocytes during all phases of inflammation – from initiation to resolution.

At the beginning of inflammation, activated tissue macrophages and dendritic cells (DCs) secrete CXCL8 (IL-8), CXCL1 and CXCL2, which primarily attract neutrophils, and CCL2 (also called monocyte chemoattractant protein - MCP-1) and CCL3/4 (also called macrophage inflammatory protein - MIP-1), which attract monocytes [10]. As neutrophils are very short-lived, new cells have to be attracted throughout the time of inflammation. Attracted activated neutrophils secrete pro-inflammatory cytokines IL-1β and TNFα, which stimulate further the production of neutrophil attracting chemokines [8]. Furthermore, metalloproteinases secreted by macrophages, neutrophils and endothelial cells can cleave chemokines such as CXCL8 and CXCL1 to increase their chemotactic potency and so amplify the signal for neutrophil infiltration [20]. Neutrophils also produce mediators such as soluble complexes of IL-6 and its receptor IL-6R, which activate endothelial cells to express adhesion molecules and chemokines (e.g. CCL2). This promotes recruitment and extravasation of monocytes [21].

During early resolution of inflammation new signaling molecules come into the play and stop the recruitment of leukocytes and induce clearance of cellular debris. For example, increased expression of the chemokine receptor CXCR5 on apoptotic neutrophils leads to sequestration of CCL3 and CCL5 and thus reduction of infiltration of new neutrophils [7]. Phagocytosis of apoptotic neutrophils by macrophages leads to their secretion of anti-inflammatory cytokines TGFβ and IL-10 [22] and release of metalloproteinases, which inactivate chemokines by cleavage. Binding of these inactive chemokines to their receptors can lead to the receptor blocking and further dampening of inflammation [7].

1.1.4 Lipid mediator signaling

Pro-inflammatory lipid mediators

Lipid mediators are further signaling molecules, which regulate inflammation. Prostaglandins and leukotrienes have been for long time appreciated for their pro-inflammatory actions [23]. They are generated in leukocytes from the essential omega-6 polyunsaturated fatty acid (PUFA) arachidonic acid through the cyclooxygenase (COX) and lipoxygenase (LOX) pathways, respectively (Figure 1.1). They act at distinct GPCRs, which are expressed on leukocytes and endothelial cells. Prostaglandin signaling leads to vasodilatation, fever and pain, leading to some of the cardinal signs of inflammation [1]. Leukotriene B4 is a potent chemoattractant of neutrophils, inducer of neutrophile granule release and of ROS production [24]. Prostaglandin synthesis is the target of probably the most widely used anti-inflammatory agent – aspirin and of further non-steroidal anti-inflammatory drugs such as indomethacin and ibuprofen. Aspirin blocks the synthesis of prostaglandins and thromboxane (TXA) by irreversible inhibition of both COX isoforms [25].

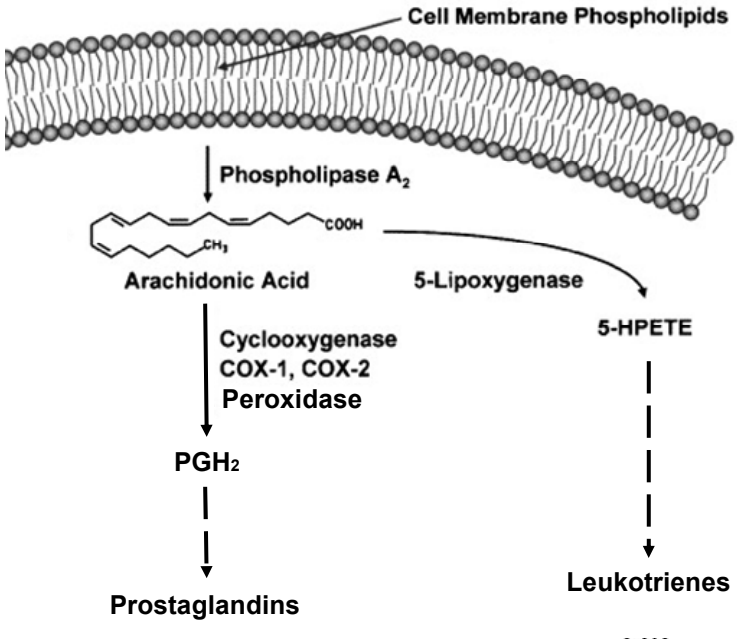

Figure 1.1: Synthesis of leukotrienes and prostaglandins

Adapted from [1]. Leukotrienes and prostaglandins are not stored within cells, but are synthesized as required in response to hormonal stimuli. First, arachidonic acid is released from the cellular phospholipids by the action of the enzyme **phospholipase A_2**. Next, the free fatty acid is converted to the precursor of prostaglandin **PGH_2** by a sequential reaction with **cyclooxygenases** (COX-1 or COX-2) and a peroxidase, or to the precursor of the leukotrienes **5-HPETE** by the **5-LOX** [26]. In subsequent reactions with series of different cell specific enzymes, prostaglandins and leukotrienes are then synthesized from their precursors.

Anti-inflammatory and pro-resolving lipid mediators

In the 1990´s, the lipoxins have been discovered as a novel class of lipid mediators, which had anti-inflammatory and pro-resolving properties [27]. Lipoxins are typically synthesized from arachidonic acid by trans-cellular pathways. The cell-cell interaction is needed because few cell types express both of the required LOXes. For example, lipoxins are produced during the interaction of infiltrating neutrophils (which express 5-LOX) and epithelial monocytes (which express 15-LOX) in inflamed target organs, or after interaction of neutrophils with platelets (which express 12-LOX) [28]. Using a murine dorsal air pouch model of spontaneously resolving inflammation, it has been documented that while amounts of LTB_4 and PGE_2 increase dramatically upon induction of an acute inflammatory response, lipoxin A_4 (LXA_4) is released in the late phase in parallel with a sharp reduction of neutrophil trafficking [29]. The same study showed that PGE_2 induces the lipoxygenase-class switching (from 5-LOX to 15-LOX activity) in neutrophils leading to reduced synthesis of LTB_4 and increased synthesis of LXA_4 [29].

An analog of the LXA_4, the 15-epi-lipoxin or aspirin-triggered lipoxin has also been shown to be synthesized through an alternative pathway after the acetylation of COX-2 by aspirin [30]. Acetylation does not completely block the catalytic activity of COX-2 but shifts it from endoperoxidase to LOX activity and COX-2 starts transforming arachidonic acid to the precursor of aspirin triggered LXA_4, 15(R)-HETE. Although the stereochemistry of the aspirin triggered LXA_4 differs from the naturally produced ones it has the same pro-resolving functions [31].

LXA_4 and aspirin triggered LXA_4, display a variety of anti-inflammatory and pro-resolving actions. They block neutrophil chemotaxis and neutrophil adhesion to and transmigration through endothelial monolayers [32,33], increase monocyte migration and non-phlogistic phagocytosis of apoptotic neutrophils by macrophages [34]. LXA_4 and aspirin triggered LXA_4 are protective in many animal models of acute or chronic inflammation, where they reduce neutrophil infiltration, increase survival and decrease the extent of tissue damage (for review see [35]).

Several further pro-resolving lipid mediators have been since isolated from inflammatory fluids taken during resolution of inflammation from animals treated with aspirin. It has been shown that the aspirin acetylated COX-2 is able to transform the omega-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) to 18-HEPE and 17-HDHA, respectively. These precursors are subsequently transformed by LOXes to resolvins and protectins. Resolvins of the E series are derived from EPA, while protectins and D series

resolvins are derived from DHA. Similarly to LXA_4, resolvins were shown to be synthetized upon cell-cell interaction (Figure 1.2). For example, aspirin treated endothelial cells were reported to convert EPA to 18-HEPE, which was be further converted to RvE1 by activated neutrophils [36]. RvE1 as well as RvD1 and protectins have been also detected in biological fluids of animals and humans not treated with aspirin, suggesting that they have physiological functions and can be synthesized without the aspirin intervention on COX-2 [37]. Resolvins of the D series and protectins were shown to be synthesized by LOX pathways, E series resolvins have been suggested to be synthesized in a pathway involving the cytochrome P-450 [38].

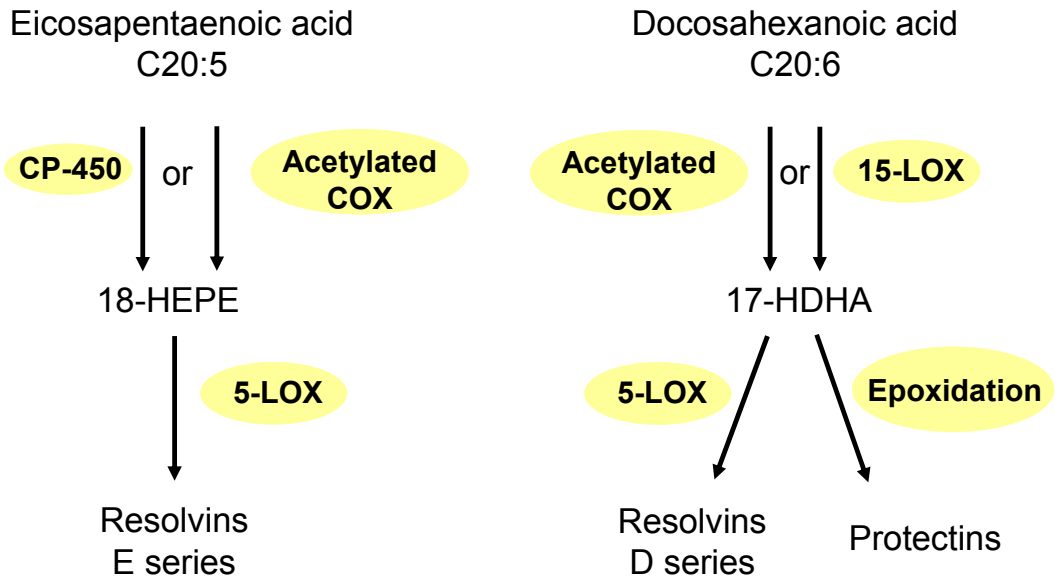

Figure 1.2: Synthesis of resolvins and protectins

In vascular endothelial cells, aspirin acetylated COX-2 transforms EPA to 18-HEPE, which is taken up by neutrophils, and further converted to E series resolvins by 5-LOX activity. Similarly, DHA is converted to 17-HDHA by acetylated COX-2 in endothelial cells, and further to D series resolvins by 5-LOX activity or to protectins by epoxidation in neutrophils. Alternatively in the absence of aspirin, 18-HEPE and 17-HDHA can be synthetized by CP-450 or 15-LOX, respectively.

Resolvin E1 and resolvin D1 have been so far studied most extensively. Similar to LXA_4, RvE1 and D1 reduce neutrophil infiltration and have been shown to be beneficial in many animal models of acute and chronic inflammation having protective function, increasing survival rates and reducing tissue damage and loss of function (for review see [39]). The beneficial effects of an elevated dietary intake of omega-3 polyunsaturated fatty acids, specifically eicosapentaenoic acid (EPA) and docosahexaenoic (DHA), are widely accepted. They have been shown to be protective in conditions associated with chronic inflammation like cardiovascular disease, arthritis, Alzheimer's disease, asthma and peritonitis as well as

components of metabolic syndrome [40,41,42,43,44,45]. However, the mechanism of their protective action had not been understood. Lipid mediators derived from EPA and DHA with their anti-inflammatory and pro-resolving properties are believed to be one of the underlying explanations for the beneficial character of omega-3 PUFA [46].

1.2 Resolvin E1

Resolvin E1 (Figure 1.3) is a lipid mediator, which was first isolated during the resolution phase of inflammation from mice treated with aspirin and EPA [36]. Given the potent pro-resolving characteristics of RvE1 similar to the actions of the lipoxins, GPCRs closely related to the ALXR were screened for RvE1 stimulated activation. The GPCR ChemR23 was identified as the receptor through which Resolvin E1 inhibited TNFα stimulated NF-κB activation in ChemR23 transfected HEK cells [47].

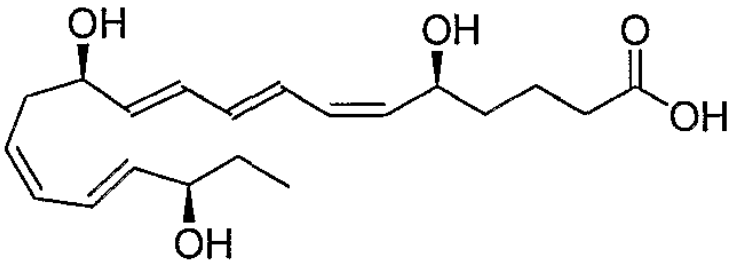

Figure 1.3: Structure of the lipid mediator Resolvin E1

5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-eicosapentaenoic acid,
source www.cyberlipid.org

1.2.1 Resolvin E1 signaling

Downstream signaling through ChemR23

Although the signaling pathway triggered upon RvE1 binding to ChemR23 in different cell types remains to be established, one study pinpointed several enzymes that could be involved in its signaling in human macrophages. Ohira et. al. showed that RvE1 induced phosphorylation of ribosomal S6 protein (rS6) in a ChemR23 dependent manner. This phosphorylation was reduced by the inhibitors of ERKs, PI3-K and mTOR kinases indicating that these enzymes are involved in the signaling cascade leading to rS6 phosphorylation. In addition, inhibiting ERK and mTOR signaling abrogated RvE1 stimulated phagocytosis in macrophages leading to the assumption that ERK, mTOR and rS6 phosphorylation are necessary for RvE1 stimulated phagocytosis [48].

Downstream signaling through alternative receptors

Next to ChemR23, RvE1 was also shown to bind the leukotriene B4 receptor BLT1 [49]. RvE1 increased intracellular calcium mobilization in human neutrophils and blocked LTB_4 mediated calcium release when given prior to LTB_4 exposure. In BLT1 transfected cells, RvE1 reduced LTB_4 dependent NF-κB activation [49]. In a mouse model of peritonitis, RvE1 reduced the number of infiltrating neutrophils. This effect could not be shown in BLT1 deficient (-/-) mice indicating that it is BLT1 dependent [49]. In another study, RvE1 accelerated apoptosis of neutrophils evoked by phagocytosis of microbial particles and enhanced NADPH oxidase-derived reactive oxygen species generation and subsequent activation of caspase-8 and caspase-3 in a BLT1 dependent manner [50]. Because ChemR23 has so far not been detected on the surface of neutrophils it is believed that all effects of RvE1 on neutrophils are BLT1 driven.

1.2.2 RvE1 in inflammation

RvE1 showed beneficial pro-resolving effects in various animal models of acute and chronic inflammation. In zymosan induced peritonitis, RvE1 increased the infiltration of mononuclear cells when given at the same time with the zymosan challenge. When given at the peak of inflammation, RvE1 reduced the numbers of infiltrating neutrophils [51]. In a model of acute lung inflammation, treatment with RvE1 at the peak of inflammation facilitated resolution by reducing neutrophil and increasing monocyte/macrophage numbers in bronachoalveolar lavage fluid [50]. In the 2,4,6-trinitrobenzene sulfonic acid induced colitis, a model for Crohn's disease, mice treated with RvE1 had an improved survival rate, lost less weight and the colonic mucosa was less affected than in not treated mice [52]. Also in the dextran sulfate sodium induced colitis, RvE1 had a beneficial impact on body weight, colon length and the state of the colon epithelium [53]. In a rabbit model of periodontitis, topical treatment with RvE1 reduced alveolar bone destruction and extremely reduced the neutrophil infiltration [54]. Furthermore, RvE1 reduced inflammation in ocular tissues by reducing the number of infiltrating macrophages [55]. Another role was recently revealed, when it was shown that RvE1 along with RvD1 and LXA_4 regulate and reduce pain [56,57,58]. Several synthetic analogs of resolvins and lipoxins are currently in clinical trials for ocular, lung, bowel and kidney inflammation [38].

The above described systemic pro-resolving effects are due to differential signaling of RvE1 in several cell types. As described above, RvE1´s potent effects on neutrophils are most likely solely elicited through the LTB1 receptor as neutrophils do not express ChemR23. In contrast,

RvE1 signaling in macrophages and DCs have been shown to be ChemR23 dependent. In macrophages, RvE1 increases phagocytosis of microbial particles as well as of apoptotic neutrophils in ChemR23 dependent manner [48,59] and thus greatly contributes to the clearance of the inflamed area. In dendritic cells isolated after injection of a pathogen extract, RvE1 blocks IL-12 secretion [47]. RvE1 was further reported to signal in platelets, where it reduced ADP-stimulated aggregation and thromboxane generation in a ChemR23 dependent manner. In human intestinal epithelial cells, RvE1 in a ChemR23 dependent manner induced the intestinal alkaline phosphatase, which can detoxify gram-negative bacteria by dephosphorylation of LPS and thus contribute to the reduction of inflammation [53].

1.2.3 RvE1 in obesity

Although RvE1´s receptor ChemR23 is highly expressed in adipocytes and was shown to play a role in adipocyte differentiation and metabolism, RvE1 signaling in adipocytes has not been studied. Resolvin E1 and its precursor 18-HEPE have been detected in human subcutaneous and perivascular adipose tissue [60]. 18-HEPE was also detected in mouse adipose tissue, where it was significantly increased in obese mice. A greater increase was observed in genetically caused obesity in leptin deficient *ob/ob* mice and a milder increase during diet induced obesity in wild type (WT) mice. RvE1 itself was detectable in mouse adipose tissue only after a n-3 PUFA treatment, which was connected with increased adiponectin in plasma, increased transcription of peroxisome proliferator-activated receptor gamma (PPARγ) and glucose transporter (GLUT-4) and improved insulin sensitivity [61]. Although another member of the resolvin family - RvD1 was shown to reduce inflammation and macrophage infiltration in obese fat and skew macrophage polarization in obese fat towards M2 phenotype [62,63], similar studies with RvE1 have not been reported.

However, systemic RvE1 treatment may have a beneficial effect on metabolic syndrome as intraperitoneal injection of RvE1 protected against hepatic steatosis in *ob/ob* mice. It decreased numbers of macrophages infiltrated in the liver and induced adiponectin, GLUT-4, insulin receptor substrate (IRS-1), and PPARγ expression in the adipose tissue indicating that RvE1 could improve insulin sensitivity in obese animals [64]. However, the cellular signaling behind these systemic changes was not followed.

1.3 Inflammation in obesity

Obesity is a growing problem world-wide. According to the WHO, obesity has reached epidemic proportions globally, with at least 2.8 million people dying each year as a result of being overweight or obese. Individuals with BMI ≥ 30 are considered obese, however, overweight with a BMI ≥ 25 can already have health damaging effects [65,66]. This is because excessive body fat is closely associated with risk factors for diseases such as type 2 diabetes (T2D), cardiovascular disease and hepatic steatosis [67]. It has been for some time recognized that obesity is connected with low level systemic inflammation, which is the driving force behind most obesity related disorders [68].

1.3.1 Possible triggers of obesity induced inflammation

The exact reason why inflammation is initiated in adipocytes with increasing fat mass is to date unknown. However, there are several hypotheses on the molecular and cellular triggers of adipose tissue inflammation. First, nutrient overload can activate pathogen-sensing receptors. For example, increased levels of free fatty acids in the circulation can be observed in obesity. It has been shown that saturated free fatty acids can trigger TLR-4 signaling and initiate inflammatory signaling in adipocytes and macrophages and induce ROS production in vascular endothelial cells [69,70]. Further, growing adipose tissue is not enough vascularized and adipocytes can be exposed to hypoxic conditions which leads to induction of inflammatory gene transcription and to cell damage and cell death [71]. Next, excessive nutrient intake leads to perpetuating ER stress of the adipocytes which ultimately leads to apoptosis of the cells [72]. The expression levels of genes involved in ER stress have been shown to be significantly upregulated in obese mice. The ER stress caused increased transcription of the inflammatory cytokines TNFα, IFN-γ, IL-1β and IL-6, and its alleviation led to suppression of these cytokines and to improvement of metabolic dysfunction [72].

1.3.2 Signs and consequences of obesity induced inflammation

The low systemic inflammation induced by and prevailing during obesity differs in many aspects from classical inflammation described above. First, it is initiated within the adipose tissue in response to excessive nutrient intake and is driven and sustained by specialized metabolic cells such as adipocytes [73]. Second, it is not accompanied by any of the cardinal signs of inflammation and it remains on a low level with modest but prevailing increase of

cytokines. Finally, obesity induced inflammation stays unresolved during the overweight state but can be reversed by weight loss [74,75].

Adipose tissue is not only an energy storage organ and fat depot. It is an active endocrine organ secreting mediators and signaling peptides regulating satiety, energy expenditure, glucose and lipid metabolism, and inflammation. During obesity, this system gets out of balance. In obese adipose tissue, the adipocytes become enlarged [76] and secrete increased amounts of TNFα [77], IL-6 and CCL2 [78]. These inflammatory cytokines and chemokines secreted by adipocytes attract increased numbers of macrophages into the adipose tissue [79]. In fact, crown-like structures of macrophages surrounding adipocytes are a typical feature of obese adipose tissue [76]. Infiltrated macrophages scavenge apoptotic adipocytes and are thought to contribute to remodeling of the adipose tissue [68]. Also a shift in macrophage polarization from M2 to M1 can be observed in obese adipose tissue [80]. As a result, obesity is connected with elevated concentration of inflammatory cytokines such as TNFα, IL-6, IL-1β and CCL2 in the circulation contributed from obese adipocytes and adipose tissue M1 macrophages [75,81]. These cytokines attract and activate further immune cells and so lead to aggravation of the inflammatory status of the tissue. At the same time lower concentrations of anti-inflammatory IL-10 and adiponectin can be observed within the adipose tissue caused by the phenotype switch in macrophages and by the inflamed nature of the adipocytes [35]. Furthermore, recent studies have provided evidence that $CD8^+$ T lymphocytes are present in obese adipose tissue and contribute to macrophage recruitment and adipose tissue inflammation [82].

One of the most common consequences of the obesity induced inflammation is insulin resistance. Inflammatory stimuli such as TNFα, secretion of which is increased in obesity, decrease insulin signaling in adipocytes and thus decrease their insulin sensitivity and subsequently glucose uptake [83]. At the same time, nutrient or inflammatory signals activate kinases such as c-jun terminal kinase (JNK) and the inhibitor of κ kinase (IKK), which target IRS-1 and inhibit the insulin receptor signaling cascades [81,84]. In addition, these kinases activate the transcription factors activator protein-1 (AP-1) and NF-κB, respectively, inducing transcription of inflammatory genes including inflammatory cytokines such as TNFα and IL-1β [68]. This leads to further augmentation of the inflammation. TNFα and IL-1β and interferons were also reported to interfere with lipid storage in adipocytes by inducing lipolysis [85] and blocking the nuclear receptor PPARγ, which regulates adipogenesis and lipid and glucose metabolism in adipocytes [86]. As a consequence of PPARγ downregulation, adiponectin expression is reduced. Adiponectin is an important inducer of

insulin sensitivity in adipose tissue, liver and skeletal muscle. Moreover, adiponectin stimulates secretion of anti-inflammatory cytokines (i.e., IL-10), blocks NF-κB activation, and inhibits release of TNFα, IL-6, and inflammatory chemokines from macrophages [87,88]. Thus obesity induced inflammation is closely intertwined with insulin insensitivity which eventually leads to the development of T2D.

In addition, obesity and the obesity associated insulin resistance are important independent risk factors for cardiovascular diseases [89]. Low systemic inflammation accompanying obesity as well as the disrupted lipid and glucose metabolism in obese adipose tissue induce and accelerate the progression of atherosclerosis [78].

1.4 Inflammation in atherosclerosis and coronary artery disease

Atherosclerosis is a condition, in which fatty streaks are created in the arterial intima and develop into atherosclerotic lesions or plaques. These plaques can obstruct the blood flow causing arterial stenosis or rupture leading to exposure of prothrombotic material from the plaque to blood. Subsequently created thrombus can cause myocardial infarction or ischemic stroke and lead to death. Atherosclerosis is the underlying cause of coronary artery disease (CAD), the leading cause of death worldwide. Obesity, hypercholesterolemia, high blood pressure, smoking and age are all factors involved in the progression of atherosclerosis. However, inflammation is an important player in atherosclerosis from the initiation of the plaque formation to its regression, or growth and final rupture [3].

1.4.1 Development of an atherosclerotic plaque

A detailed understanding of the cellular and molecular events from initiation of atherosclerosis to plaque progression and rupture has been acquired using apoE and low density lipoprotein (LDL) receptor ($^{-/-}$) mice, which develop atherosclerosis spontaneously or after high fat diet, respectively. Atherogenesis is initiated by the infiltration of LDL particles into the intima of the arterial wall [90]. Hypercholesterolemia and sheer stress increased by high blood pressure lead to local activation of arterial endothelium [91,92]. As a result, endothelial cells express adhesion molecules (e.g. V-CAM1) and leukocyte chemoattractants (e.g. CCL2 and CCL5) [93], which enable the adhesion of monocytes and T-cells to the endothelium and their subsequent extravasation into the intima. Consequently, LDLR$^{-/-}$ mice expressing truncated V-CAM1 show decreased early lesion formation [94]. Once in the intima, monocytes differentiate into macrophages and scavenge oxidized LDL particles [95]. The center of the plaque becomes filled with such lipid laden macrophages called foams cells

(Figure 1.4). LDL cholesterol uptake by macrophages leads to their activation and release of inflammatory mediators, which attract further monocytes into the lesion and activate the present T-cells. The cytokines secreted by activated T-cell then contribute to the inflammation in the plaque. For example, IFN-γ, a cytokine secreted by Th1 cells, is present in the human plaques and has pathogenic effects, such as enhanced protease and chemokine secretion, upregulation of adhesion molecules, induction of pro-inflammatory cytokines, and enhanced activation of macrophages and endothelial cells [96]. Unresolved inflammation leads not only to propagation of the plaque but can also negatively influence its stability. For example, IFN-γ inhibits proliferation of smooth muscle cells, which stabilize the plaque cap [97]. The lack of IFN-γ receptor apoE$^{-/-}$ mice leads to inhibition of atherogenesis [98]. In addition, matrix metalloproteinases (MMPs) released by activated macrophages and vascular smooth muscle cells degrade the collagen in the cap thus compromising its stability and leading to formation of so called vulnerable plaque [99].

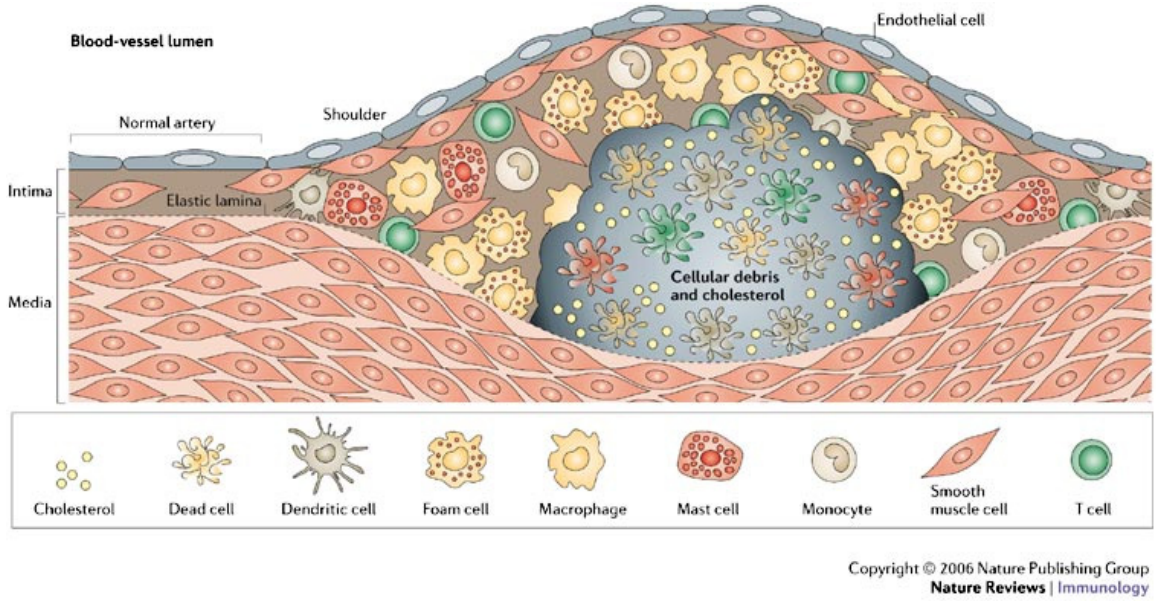

Figure 1.4: Cellular composition of an atherosclerotic plaque.

The core of an atherosclerotic plaque contains free lipids and apoptotic cells. These are surrounded by immune cells including macrophages, lipid laden foam cells, T-cell and in lower numbers B-cells, mast cells and dendritic cell. A fibrous cap consisting of smooth muscle cells and collagen fibers stabilizes the plaque. Taken from [90].

1.4.2 Atheroprotective role of anti-inflammatory and pro-resolving mediators

Lesion progression and its development into a vulnerable plaque is fueled by impaired resolution of inflammation within the plaque. There is evidence that anti-inflammatory and pro-resolving signaling molecules could stop or even reverse the process. The anti-inflammatory cytokines IL-10 and TGFβ have been shown to have great atheroprotective effects. IL-10 acts on macrophages, where it induces the suppressor of cytokine signaling (SOCS3) and inhibits the NF-κB pathway. This leads to reduced secretion of inflammatory cytokines and metalloproteinases, and to the suppression of macrophage mediated T-cell activation. In addition, IL-10 has been shown to regulate the uptake and efflux of cholesterol in macrophages [100]. $LDLR^{-/-}$ mice systemic overexpression of IL-10 as well as overexpression in macrophages protected from atherosclerosis [100], while Il-10 deficiency exacerbated atherosclerosis [101]. TGFβ suppresses inflammation by modulating T cell activation and promotes collagen production thereby stabilizing the plaque [102]. TGFβ deficiency in $apoE^{-/-}$ leads to increased infiltration of inflammatory macrophages and Th1 cells and to reduced collagen content in the plaque [103]. IL-10 and TGFβ are secreted by several cell types including Th2 and regulatory T cell (T-reg) and M2 macrophages. Induction of T-reg cells by vitamin D3 or by anti-CD3 antibody treatment have been shown to decrease atherosclerosis in $apoE^{-/-}$ mice [104,105]. On the other hand, treatment with thioredoxin shifted the macrophage phenotype of lesion macrophages toward M2 and was atheroprotective in ApoE knock-in mice [106]. T-reg cell activation or re-polarization of macrophages toward the M2 state within the plaque could therefore represent a new strategy to combat atherosclerosis [103].

The lipid mediators lipoxins and resolvins have been reported to stimulate resolution of inflammation in many animal models of chronic inflammation [33] and there are hints that they are also atheroprotective. Positive effects of individual mediators on several cell types contained within the plaques have been shown in cell culture. LXA_4 reduced inflammatory cytokine secretion in macrophages, and CCL2 and P-selectin expression in endothelial cells [107]. In addition, LXA_4 and RvE1 inhibit vascular smooth muscle cell migration [108]. As the synthesis of these lipid mediators increases after aspirin treatment, they might offer an additional explanation for beneficial effects of aspirin treatment in atherosclerosis and CAD [30,109].

1.4.3 Biomarkers of inflammation in atherosclerosis

Atherosclerotic plaque is not devoid of the influences from the rest of the body and inflammation in other organs and tissues (such as the obesity triggered inflammation in adipose tissue) can potentiate plaque progression. Likewise, the inflammation within the plaque is not isolated but is reflected by elevated inflammatory markers in the circulation. Many inflammatory pathways are implicated in atherosclerosis and the products of several of them can be measured and used in the assessment of risk for cardiovascular events. Such risk assessment helps to identify high-risk individuals, to diagnose the disease conditions and make a prognosis.

C-reactive protein (CRP), is an acute-phase protein secreted by the liver in response to the inflammatory cytokines IL-6, TNFα and IL-1β originating at the site of inflammation. Modest elevation of CRP can be observed already in preclinical states of atherosclerosis and it is a strong independent predictor of cardiovascular events [110]. In addition, other inflammatory markers can indicate the state of the disease. Increased levels of the soluble adhesion molecules VCAM and ICAM are observed in patients with acute coronary syndromes and CAD [111], while MMPs have been reported to be elevated in patients following myocardial infarction and have been suggested as marker of unstable plaques [112]. Finally, the balance between pro- and anti-inflammatory cytokines has emerged as a major determinant of plaque stability. Increased levels of inflammatory cytokines IL-6, IL-1β, IFN-γ and TNFα and reduced levels of the anti-inflammatory IL-10, TGFβ and adiponectin have been reported to be associated with CAD and myocardial infarction [111].

1.5 The receptor ChemR23

1.5.1 ChemR23 gene and transcript

ChemR23, also named chemokine-like receptor 1 (CMKLR1), is a GPCR specifically binding the peptide chemerin and the lipid mediator resolvin E1. It was indentified in late 1990´s as an orphan receptor and localized to the region q24.1 of chromosome 12 [113], [114]. ChemR23 has 7 membrane spanning domains typical for GPCRs from the rhodopsin family. It is most closely related to chemoattractant receptors and to the formyl peptide receptors, not, as its second name suggests, to the chemokine receptors. It has high homology to the second chemerin receptor GPR1 [115], and the receptors for the complement fragments C3a and C5a (Figure 1.5).

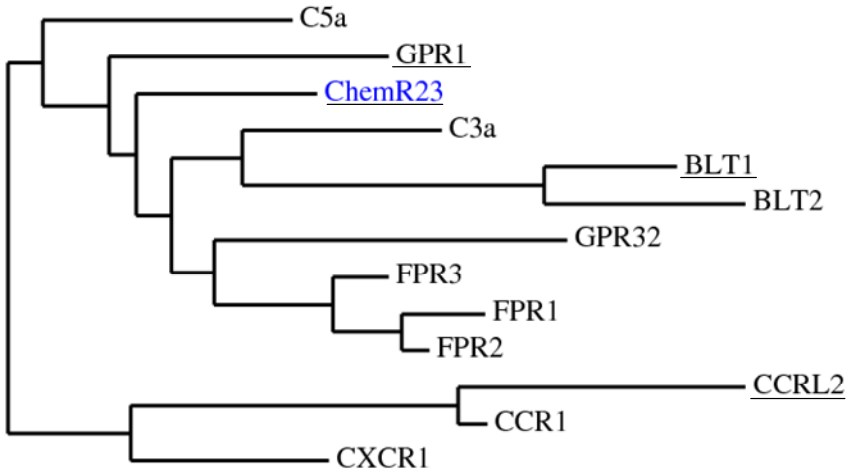

Figure 1.5: Phylogenetic tree illustrating the structural similarities between ChemR23, other receptors for leukocyte chemoattractants and selected chemokine receptors.

Receptors sharing a ligand with ChemR23 are underlined. The tree was created with www.phylogeny.fr.

Similarly to other chemoattractant receptors, the ChemR23 gene constitutes of a single coding exon and at least one non-coding exon with a large intron separating the start points of transcription and translation [116]. The cDNA sequences of the human ChemR23 deposited in GenBank indicate that it is alternatively spliced. The promoter regions and the transcription regulation of the different splicing variants in individual cell types have however not been studied. The mouse orthologue of ChemR23 named also DEZ was cloned by Methner et al [117] and was localized to the mouse chromosome 5 [118]. The genomic region of the mouse ChemR23 orthologue has been closely studied. The ChemR23 gene is transcribed from 2

alternative promoters in different cell types and is differently spliced. Both identified promoters lack the TATA box [118,119].

1.5.2 ChemR23 expression

Transcripts of ChemR23 were first detected by Northern Blotting in the placenta, lung, liver and hematopoietic tissues such as spleen, lymph nodes and thymus. Because of the receptor's homology to chemoattractant and formylated peptide receptors, and the high expression in hematopoietic and lymphoid tissues, ChemR23 was first closely studied in leukocytes. ChemR23 is expressed on circulating monocytes and monocytes derived macrophages [114], on subsets of NK cells [120], platelets [121], on circulating dendritic cells and on plasmacytoid and myeloid dendritic cells within the lymph nodes but not in Langerhans cells (dendritic cells resident in the healthy skin) [122]. In leukocytes, ChemR23 was shown to constitute a minor co-receptor for a subset of human immunodeficiency viruses [114,123]. Later, ChemR23 was shown to be also expressed on other different cell types - adipocytes and preadipocytes [124], human endothelial cells [125], joint chondrocytes [126] and skeletal muscle cells [127] and vascular smooth muscle cells [108].

1.5.3 ChemR23 regulation

To date, little is known about the regulation of ChemR23 during different stages of the immune response. Several groups have studied the changes in ChemR23 transcription and protein expression in leukocytes in response to inflammatory or anti-inflammatory stimuli. In human monocytes, ChemR23 mRNA levels increased after stimulation with the inflammatory cytokines TNFα and IFN-γ [47]. In human macrophages and DCs, low concentrations of LPS (10 ng/ml) decreased transcription of ChemR23, while higher concentrations (1 ug/ml) moderately increased ChemR23 transcripts as compared with untreated cells [114]. In NK cells, ChemR23 was strongly downregulated after long-term (4-6 days) stimulation with IL-2 and IL-15 - cytokines responsible for the activation of NK cells in terms of cytotoxicity and cytokine production [120]. This would suggest that NK cells express ChemR23 only during the early phase of migration toward the inflamed tissue. In endothelial cells, ChemR23 is upregulated by inflammatory cytokines (IL-1β, TNFα, IL-6) [125]. Overall, it seems that ChemR23 is upregulated by inflammatory stimuli, which would hint to its function during the early stages of inflammation.

In mice, ChemR23 was detected on monocytes, macrophages and DC progenitors but not on most DCs. In mouse macrophages, ChemR23 was downregulated by inflammatory stimuli

such as TLR ligands (LPS, CpG) and inflammatory cytokines, and upregulated by TGFβ [128]. In another study, ChemR23 was downregulated in peritoneal exudate cells after an overnight treatment with IFN-γ and LPS but not with IL-4 or IFN-γ alone [129]. The authors of these studies suggest that ChemR23 has an anti-inflammatory role. It remains unclear why the regulation differs in mice and human and whether it is possible to ascribe to ChemR23 a clear inflammatory or anti-inflammatory function.

1.5.4 ChemR23 deficiency in mouse models

Several ChemR23$^{-/-}$ mouse models have been reported. Although ChemR23 is present on the surface of many different cell types and was shown to have a role in leukocyte recruitment, adipogenesis and β-cell function as is in detail described below, ChemR23$^{-/-}$ mice exhibit no obvious difference in phenotype to WT mice. Under normal conditions, ChemR23$^{-/-}$ mice are generally healthy, fertile and have no immune abnormalities [130,131,132,133,134]. The lack of ChemR23 only becomes apparent in experimental models of acute or chronic inflammation and upon metabolic challenges. ChemR23$^{-/-}$ mice exhibited increased accumulation of neutrophils and macrophages in lungs after LPS-induced lung insury [133]. However, decreased accumulation of macrophages and smaller inflammatory lesions in the central nervous system was observed in a mouse model of multiple sclerosis [130]. In addition, reduced food consumption, body mass and adiposity was reposted in ChemR23 $^{-/-}$ deficient mice by Ernst et. al.. Lack of ChemR23 was also associated with lower blood glucose, insulin, leptin and adiponectin levels, and reduced glucose tolerance in diet induced obesity [134].

1.6 Chemerin

Chemerin was identified as a ligand of ChemR23 by testing fractions of human inflammatory fluids [135], and by screening of a hemofiltrate peptide library [136] for biological activity specific for ChemR23 expressing cells. The active molecule was subsequently characterized as the product of the human Tig-2 (tazarotene-induced 2) gene previously described as a retinoid-responsive gene in psoriatic skin lesions [137].

1.6.1 Chemerin expression and isoforms

Chemerin is synthesized as a 163 amino acid (AA) long pre-proprotein, which is cleaved on the N-terminus in the cell and secreted as a 143 AA long inactive prochemerin (Chem 21-163) [135,136]. Prochemerin can be activated through the cleavage of its C-terminus by proteases of the coagulation and fibrinolytic cascades, and by proteases released from activated

neutrophils or mature adipocytes [138,139]. Active chemerin isoforms have been detected in inflammatory and other biological fluids (Table 1.1) and in minor quantities in plasma. The inactive prochemerin was found to circulate in blood [140].

Table 1.1: Biological sources of identified chemerin isoforms

Ascites [135]	Chem 21-157
Hemofiltrate [136]	Chem 21-154
Cerebrospinal fluid [141,142]	Chem 21-158
Plasma [139,140,141,142]	Chem 21-155, -157, -158, -163
Synovial fluid [142]	Chem 21-158

Chemerin is mainly expressed by the liver, white adipose tissue, and placenta [124,143] and to a lesser extend by many other tissues such as lungs, brown adipose tissue, heart, kidney, skeletal muscle, pancreas [144] and platelets [145]. Liver and white adipose tissue are believed to be the main source of circulating chemerin [146].

The degree of chemerin activity depends on the exact cleavage by each of its processing enzymes [139]. Some proteases were found to cleave chemerin at more than one site and it was also suggested that some chemerin isoforms can be consecutively cleaved by different proteases thereby modulating the degree of their activity. Chemerin activation and function is believed to be regulated specifically in different tissues and at different time-points depending on the expression and activity of chemerin processing enzymes. The activity of different chemerin isoform has been studied *in vitro* by several groups. Figure 1.6 gives an overview of chemerin processing enzymes, and of the resulting chemerin isoforms and their activity.

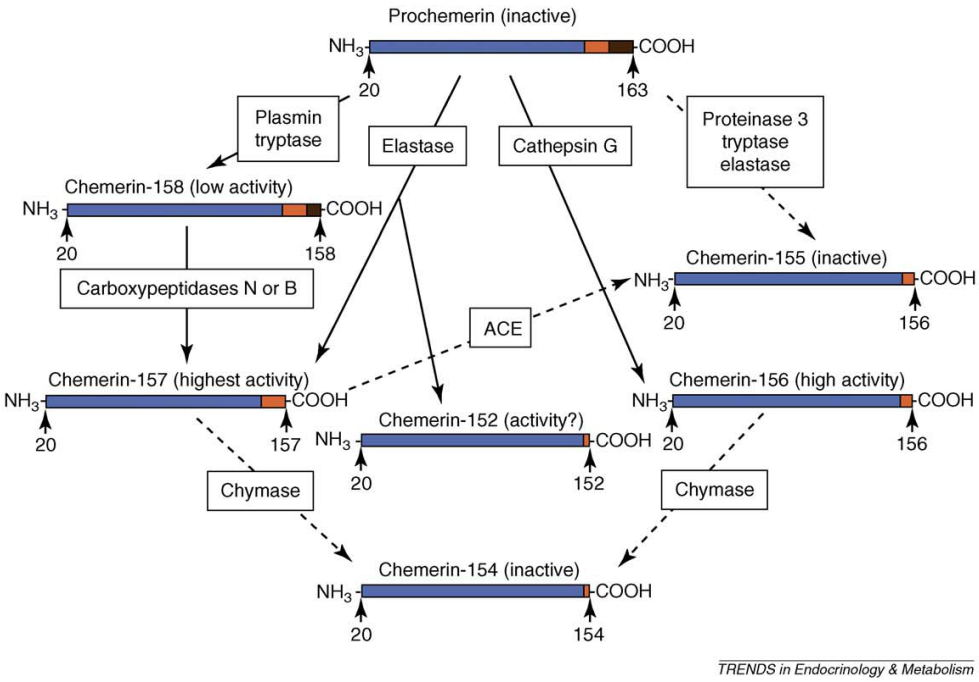

Figure 1.6: Overview of chemerin processing and activity of individual isoforms

The inactive prochemerin (AAs 20 – 163 of pre-prochemerin) can be cleaved by different proteases (white boxes) leading to isoforms of different lengths and varying activities. For example, cleavage of 6 C-terminal AAs by leukocyte elastases or sequential cleavage by plasmin and carboxypeptidase N or B results in Chem-157 with the highest activity. Subsequent cleavage of 3 AAs by mast cell chymase leads to inactivation. Elastase and tryptase have also been reported to generate Chem-155, which has no activity itself but is capable of competing with more potent isoforms thus blunting their effect. Taken from [146]

In addition, several studies have shown that synthetic chemerin-derived peptides consisting of the C-terminal 9-15 AAs alone have considerable activity. On the other hand, fragments of the mid- and N-terminal parts of the sequence do not seem to be essential for the degree for chemerin activity [131,133,147,148]. The C-terminal nonapeptide is the shortest chemerin derived peptide retaining chemerin specific activity [133,147]. Its sequence is highly conserved in all mammalian species and it has a high affinity to ChemR23 [133]. It is used in *in vitro* studies instead of full length chemerin, because the production of the recombinant chemerin proved to be a tedious process [147].

1.6.2 Chemerin signaling

Downstream signaling through ChemR23

The exact downstream signaling pathways triggered upon chemerin binding to its receptor in different cell types still have to be elucidated. In early studies, chemerin treatment was shown to trigger intracellular calcium release, phosphorylation of the p42 (ERK1) and p44 (ERK2)

MAP kinases and the inhibition of cAMP accumulation in ChemR23 transfected CHO-K1 cells [135]. Chemerin stimulation also lead to ERK1/2 phosphorylation in human endothelial cells, adipocytes, chondrocytes and fibroblast-like synoviocytes [124,125,126,149]. In human endothelial cells and chondrocytes, and in mouse macrophages, chemerin was also shown to induce the p38 MAP kinase and Akt signaling [129,149].

Downstream signaling through alternative receptors

In addition to ChemR23, chemerin binds two other GPCRs – the chemokine (CC motif) receptor-like 2 (CCRL2) and the GPR1.

CCRL2 is expressed on endothelial cells [150], adipocytes [151] and in most hematopoietic cell types [152]. Chemerin binding to CCRL2 does not trigger ligand internalization or any signaling inside the cell. Instead, CCLR2 binding accumulates chemerin on the cells surface and thus increases local chemerin concentration and availability [153].

GPR1 was reported to bind chemerin with comparable affinity to ChemR23 [115]. In pigs, the GPR1 orthologue was shown to be expressed mainly in liver, spleen, kidney and adipose tissue [154]. There is however nothing known about the function and signaling of this receptor.

1.6.3 Chemerin in inflammation

Chemerin was initially shown to act as a chemoattractant of ChemR23 expressing DCs, macrophages [135] and NK cells [120]. Active chemerin, capable of attracting ChemR23 expressing DCs *ex vivo*, was detected in psoriatic skin lesions [155]. Chemerin is found to be upregulated within inflamed tissues such as psoriatic skin and lupus lesions and in arthritis [122,135,149], and chemerin and infiltrating ChemR23 positive DCs or NK cells were also detected in oral lichen planus biopsies [120], kidney biopsies from patients with lupus nephritis [156] and in skin lupus lesions [122]. It is therefore believed that chemerin contributes to the recruitment of ChemR23 expressing leukocytes to these inflamed tissues.

In some studies, chemerin was additionally reported to have a direct pro-inflammatory role. It enhanced the secretion of pro-inflammatory cytokines such as IL-6, IL-8, TNFα, CCL2 and IL-1β [126,149] in cultured human chondrocytes and fibroblast-like synoviocytes *in-vitro*. These findings support the notion that chemerin may have pro-inflammatory properties. Conversely, inflammatory cytokines increased chemerin secretion in some cell types: TNFα increased total and bio-active chemerin secretion in human and murine adipocytes, and in

human intestinal epithelial cells and fibroblast-like synoviocytes [149,157,158]. IL-1β increased total chemerin secretion in adipocytes [159].

Elevated chemerin levels in the circulation are associated with several inflammatory diseases. Significantly elevated plasma chemerin levels were reported in patients with Crohn's disease and ulcerative colitis [160], chronic kidney disease [161] and chronic pancreatitis [162]. Plasma chemerin levels are positively correlated with CRP, the pro-inflammatory adipokines leptin and resistin [143,163,164,165], and with the levels of the inflammatory cytokines TNFα and IL-6 [165], and negatively correlated with the anti-inflammatory adipokine adiponectin [166,167]. In rheumatoid arthritis patients, anti-TNF therapy decreased circulating chemerin levels together with decreasing IL-6 levels and disease activity parameters [168].

However, it is dificult to interpret these increased chemerin levels in terms of possible functionality of chemerin in these diseases. Although the ratio of active to non-active chemerin would be decisive for its final signaling, most studies measure total chemerin level not distinguishing between individual isoforms. Therefore, the significance of elevated chemerin levels in circulation and in inflammatory fluids, and their impact on the disease are uncertain. For example in renal epithelial cells TNFα treatment *in vitro* significantly downregulated chemerin secretion. However, the supernatants of the TNFα treated cells attracted plasmacytoid DCs, in a ChemR23 dependent manner, more potently than supernatants from untreated cells [156]. This suggests that although TNFα reduces total chemerin secretion in renal epithelial cells, it at the same time promotes pro-chemerin cleavage and activation.

Animal models of inflammation

Controversly, in animal models of acute and chronic inflammation, chemerin was reported to have both pro and anti-inflammatory actions. In a mouse model of LPS-induced acute lung inflammation, chemerin reduced neutrophil infiltration and dampened inflammatory cytokine increase in bronchoalveolar lavage fluid [133] in a ChemR23 dependent manner. Chemerin treatment prior to M1 polarization with LPS and IFN-γ, inhibited inflammatory cytokine secretion (TNFα, IL-1β, IL-6 and IL-12) and induced the transcription of the anti-inflammatory TGFβ and IL-10 in mouse peritoneal macrophages. The described effects were completely ChemR23 dependent and were not seen in macrophages isolated from ChemR23$^{-/-}$ mice [131]. However, when an independent group repeated the experiment, chemerin pre-stimulation did not significantly affect the cytokine production in neither murine nor human LPS/IFN-γ stimulated macrophages [169]. Some stable chemerin derived peptides also proved to be anti-inflammatory. For example, the administration of a 15 AA long peptide (C15)

derived from chemerin´s C terminus prior to zymosan challenge protected mice against zymosan-induced peritonitis. The peptide C15 reduced the numbers of infiltrating monocytes and neutrophils and diminished peritoneal expression of inflammatory cytokines. In contrast, C15 could not suppress zymosan induced leukocyte recruitment in ChemR23$^{-/-}$ mice indicating that these effects were ChemR23 dependent [131]. C15 was also reported to increase murine macrophage phagocytosis of microbial particles and apoptotic cells *in vitro* and to enhance the clearance of microbial particles and apoptotic neutrophils in zymosan-induced peritonitis [148].

Some of the above described anti-inflammatory effects of chemerin are most probably due to chemerin´s action on non-leukocytic cells, such as epithelial or endothelial cells, where there is indication that chemerin could induce rescue pathways and reduce leukocyte cell adherence. In epithelial cells, chemerin increased the intestinal alkaline phosphatase expression *in vitro* leading to detoxification of E. coli cells [53] and in human vascular endothelial cells, chemerin attenuated TNFα-induced VCAM-1 expression, leading to decreased monocyte adhesion to the endothelial cells [170].

On the other hand, chemerin signaling seems to be required for the initiation of inflammation by attracting leukocytes. For example, ChemR23$^{-/-}$ mice develop milder experimental autoimmune encephalomyelitis likely due to lower macrophage counts in the lesions [130]. Inflammation caused by chronic cigarette smoke exposure lead to increased chemerin release in the lung epithelium of mice. This inflammation was attenuated in ChemR23$^{-/-}$ mice through reduced recruitment of inflammatory neutrophils and monocytes [132]. When administered to murine macrophages, chemerin promoted their adhesion to the extracellular matrix protein fibronectin and to the adhesion molecule VCAM-1, supporting a role in the recruitment and retention of leukocytes at sites of infiltration [129].

In summary, chemerin was reported to trigger both inflammatory and anti-inflammatory signals depending on the cell type it acts on and on the proteolytic cleavage it undergoes. It remains to be established in which moment of inflammation chemerin is most essential – initiation or resolution.

1.6.4 Chemerin in adipose tissue and obesity

In 2007 chemerin was identified as an adipokine. Chemerin secretion is induced during differentiation of pre-adipocytes to mature adipocytes and chemerin signaling through ChemR23 on preadipocytes was shown to be essential for adipocyte differentiation [124,143]. Chemerin or ChemR23 shRNA knockdown in preadipocytes block their differentiation and

affect expression of proteins involved in glucose and lipid metabolism such as the glucose transporter GLUT4, hormone sensitive lipase and the insulin receptor [124]. Chemerin signaling was also shown to be essential during the early clonal expansion phase of adipocyte differentiation [171]. Consistent with this, a loss of ChemR23 function in mice is associated with reduced body mass due to reduced body fat, and with resistance to diet-induced obesity [134] suggesting that chemerin-ChemR23 signaling is essential for adipogenesis *in vivo*. Chemerin expression in preadipocytes is positively regulated by PPARγ [171] – an important regulator of adipogenesis and adipocyte metabolism.

In both man and mice, chemerin plasma levels are elevated in obese individuals. Chemerin levels are correlated with BMI and the waist-hip ratio in humans [143,172] and decrease after a bariatric surgery in parallel to the decrease of body fat mass [166]. This elevated chemerin is believed to originate from adipose tissue [173]. This notion is supported by reports that adipose tissue explants from obese individuals secrete significantly more chemerin than those isolated from lean individuals, and this secretion correlates with increased BMI, waist to hip ratio and fat cell volume [127]. Obesity is commonly associated with low systemic inflammation and chemerin has been suggested to be a link between obesity and inflammation [146]. Considering chemerin´s function as a chemoattractant of macrophages, increased chemerin production in the obese adipose tissue [127] might be responsible for the observed excessive infiltration of macrophages [79]. In line with this, chemerin concentrations in obese patients positively correlate with the amount of macrophages in the omental adipose tissue [172] and mice lacking chemerin have less macrophages infiltrating into the adipose tissue [144]. Furthermore, macrophage secreted inflammatory cytokines TNFα and IL-1β are positive regulators of chemerin secretion by adipocytes [157,159] possibly creating a vicious cycle of activation.

Elevated chemerin levels have been observed in individuals with the metabolic syndrome and are correlated with its markers such as low HDL cholesterol, and high blood pressure [143,163]. In mouse models, chemerin was reported to affect glucose tolerance and insulin secretion [134,174]. Therefore, it was surmised that chronically elevated chemerin levels in obesity might exacerbate already present glucose metabolism impairments and further contribute to the development of metabolic syndrome [173].

1.6.5 Chemerin in atherosclerosis

As inflammation and obesity are important risk factors for coronary artery disease, chemerin could play a role in the pathology of this disease. Although chemerin levels are associated

with several risk factors for CAD such as chronic inflammation and obesity, systemic overexpression of human chemerin in mice lacking the LDL-receptor did not affect the extent of atherosclerosis investigated by *en face* analysis of the entire aorta [175]. In humans, several small case-control studies have been carried out showing contentious results for the association of chemerin plasma levels with CAD. While 2 studies in Asians showed an association of plasma chemerin levels with atherosclerotic diseases [176,177] a third study in Caucasians showed no association [165].

1.6.6 Chemerin and RvE1 binding to ChemR23

Curiously, the two ligands of ChemR23, chemerin and RvE1 have been shown to elicit different responses upon binding the receptor in ChemR23. In a study by Arita et. al., RvE1 and a 12-AA fragment derived from chemerin competed for ChemR23 binding suggesting an identical binding site. However, in ChemR23 transfected HEK cells, the chemerin peptide increased extracellular acidification rates, while RvE1 had no effect. In contrast, both the chemerin peptide and RvE1 inhibited TNFα induced NF-κB luciferase activity. The authors concluded that RvE1 and chemerin use different signaling pathways through ChemR23 [47]. Other studies suggest that some chemerin peptides have the same effects as RvE1. For example, in human intestinal epithelial cells, both chemerin and RvE1 induced the intestinal alkaline phosphatase in a ChemR23 dependent manner [53]. This would argue for an identical pathway triggered by both ligands. However, the exact downstream signaling was not studied. The C15 chemerin peptide was reported to induce macrophage phagocytosis in murine cells in ChemR23 dependent manner, an effect also ascribed to RvE1 [148]. The effect could be blunted by an inhibitor of Syk tyrosine kinase in macrophages, which is required for lysosome phagosome fusion [148]. On the other hand, RvE1 stimulated macrophage phagocytosis engaged the Akt, ERK and mTOR kinases [178]. It is therefore likely that RvE1 and different chemerin peptides trigger different signaling pathways in different cell types. In addition, even within one cell type, binding of these ligands to ChemR23 could lead to initiation of different signaling outcomes. In GPCRs, ligand binding leads to phosphorylation, which mediates uncoupling from the G-protein but can also drive G-protein independent signaling [179,180]. It has been recently shown that the site of this phosphorylation is cell type and ligand specific, and that by adopting a specific phosphorylation profile the receptor could initiate a particular pathway [181] .

1.7 Significance of studying ChemR23

The prevalence of obesity is growing world-wide. According to the WHO more than 1.4 billion adults are obese globally and the number of children who are overweight is rapidly growing. Obesity is associated with secondary disorders such as cardiovascular disease and type-2 diabetes, which are accelerated by impaired cellular lipid and glucose metabolism, and by chronic low grade inflammation prevalent in obesity [68]. ChemR23-chemerin signaling is implicated in inflammation as well as in adipogenesis and adipocyte metabolism [173]. The ChemR23-chemerin axis may therefore represent an important link between inflammation, obesity and obesity related disorders.

The second ligand for ChemR23 is the lipid mediator RvE1, a metabolite of the omega-3 fatty acid EPA. RvE1 is one of the mediators thought to mediate the protective effects of dietary EPA [46]. Although RvE1 has established pro-resolving properties in acute inflammation models, its action in different stages of inflammation and its role in chronic inflammation is largely unknown.

Better understanding of the regulation of ChemR23 expression in different subsets of monocytes and macrophages will help to pinpoint the phase of inflammation, in which ChemR23-RvE1 and ChemR23-chemerin signaling is relevant. In addition, investigating polymorphisms in ChemR23 for an association with CAD, may implicate these two signaling pathways as players in atherogenesis and may identify ChemR23 as a potential new drug target.

2 Results

2.1 ChemR23, the receptor for chemerin and resolvin E1 is expressed and functional on M1 but not on M2 macrophages

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2.1.1 Abstract

ChemR23 is a G-protein coupled receptor expressed on monocytes, macrophages, dendritic and natural killer cells. Two ligands signal through ChemR23 – the peptide chemerin and the eicosapentaenoic acid derived lipid mediator resolvin E1 (RvE1). Chemerin acts as a chemoattractant for ChemR23 expressing leukocytes, while RvE1 promotes resolution of inflammation by induction of macrophage phagocytosis of microbial particles and apoptotic neutrophils. Although ChemR23 mediated signaling plays a role in mononuclear cell immigration to inflamed tissue as well as in the resolution of inflammation, its regulation in different polarization states of macrophages is largely unknown. We analyzed the expression and function of ChemR23 in monocytes and resting and differently activated human primary macrophages. Using 5′ RACE, we identified 3 transcription start sites and several splice variants of ChemR23 in both monocytes and macrophages. While the promoters P1 and P3 are used equally in unpolarized macrophages, stimulation with LPS or IFN-γ leads to increased transcription from P3 in inflammatory M1 macrophages. Such ChemR23 expressing M1 macrophages are chemotactic to chemerin and increase IL-10 transcription in response to RvE1 stimulation. In contrast ChemR23 is not expressed on M2 macrophages, which are also not responsive to chemerin. These results show that ChemR23 is tightly regulated in response to inflammatory and anti-inflammatory stimuli. The high expression of ChemR23 in naive and M1 macrophages supports the role of ChemR23 in the attraction of macrophages to inflamed tissue by chemerin and in the initiation of resolution of inflammation through RvE1 signaling in human macrophages. In contrast, the role of ChemR23 mediated signaling is limited in M2 macrophages.

2.1.2 Introduction

Macrophages are key players in innate immunity with an essential role in inflammatory processes [1]. Due to their phenotype diversity and plasticity, macrophages participate in all stages of inflammation from pathogen recognition to pathogen elimination, and finally to the resolution of inflammation [2]. The phenotype of macrophages changes in response to stimuli from the environment. Upon stimulation with the Th1 cytokine IFN-γ or TLR-4 ligands, macrophages undergo classical M1 activation resulting in high secretion of inflammatory cytokines such as TNFα and IL-1β, and strong microbicidal activity mediated by reactive oxygen and nitrogen species [3]. In contrast, the Th2 cytokines IL-4 and IL-13 lead to alternative M2 activation of macrophages. These macrophages are characterized by high secretion of anti-inflammatory IL-10 and IL-1RA, and high expression of the mannose and galactose receptors [4]. The M1 phenotype is associated with initiating and progressing inflammation, while M2 macrophages are implicated in tissue repair, wound healing and parasite infections [3]. In addition, it was shown that macrophages can to some extend switch from one activation state to the other [5,6] and intermediate phenotypes have been identified in specific stages of the immune response such as resolution of inflammation [7].

ChemR23, also known as the Chemokine-like Receptor 1 (CMKLR1), is a G-protein coupled receptor expressed on monocytes and macrophages, dendritic [8] and natural killer cells [9] as well as on adipocytes [10] and endothelial cells [11]. It binds two ligands - the peptide chemerin [12], and the eicosapentaenoic acid (EPA) derived lipid resolvin E1 (RvE1) [13]. Chemerin is present in high amounts in inflammatory fluids [14], was shown to attract ChemR23 expressing leukocytes [12,15], and to promote adhesion of macrophages to extracellular matrix proteins [16]. In addition, chemerin was recently discovered to be an adipokine [10]. Secreted by mature adipocytes, it stimulates preadipocytes to differentiation. Elevated serum levels of chemerin have been associated with chronic inflammatory diseases [17] [18], the metabolic syndrome and obesity [19]. There is indication that high chemerin production in obese adipose tissue [20] might contribute to the increased infiltration of macrophages observed in obese adipose tissue leading to low level inflammation [21].

The second ligand of ChemR23, RvE1, promotes resolution of inflammation in animal models of acute and chronic inflammation [22]. On the cellular level, RvE1 blocks TNF-α induced NF-κB signaling [13] and enhances phagocytosis of bacterial particles and apoptotic neutrophils by human macrophages in a ChemR23 dependent manner [23]. RvE1 is postulated to be one of the mediators of the beneficial effects of dietary EPA, which is thought to have protective function in conditions associated with chronic inflammation [24].

The above described findings indicate that the receptor ChemR23 plays a role during monocyte/macrophage recruitment to the inflamed tissue as well as during the resolution of inflammation. With the aim to better understand which macrophage phenotype responds to signals triggered through ChemR23, we analyzed the expression and function of ChemR23 in resting and differently activated human primary macrophages. We show that ChemR23 is expressed in monocytes and macrophages and is upregulated in M1 macrophages, which are chemotactic to chemerin and increase IL-10 transcription in response to RvE1 stimulation. In contrast, M2 macrophages do not express the receptor. We further show that ChemR23 is transcribed from 2 promoters in monocytes and macrophages. While promoters P1 and P3 are used equally in unpolarized macrophages, the increased transcription in inflammatory M1 macrophages is driven from promoter P3.

2.1.3 Materials and Methods

Materials

The recombinant human cytokines IL-13, IL-1β, TNF-α, TGF-β, and IFN-γ; the TLR-4 ligand LPS and the TLR3 ligand poly I:C were purchased from Sigma Aldrich (Buchs, Switzerland). IL-4 and IL-6 were purchased from R&D Systems Europe (Abingdon, U.K.). The TLR9 ligand, CpG, was synthesized by Microsynth (Balgach, Switzerland). The TLR7 and 8 synthetic ligands 3M001 and 3M002 were purchased from 3M Pharmaceuticals (St. Paul, Minnesota, USA). Chemerin (149-157) was purchased from AnaSpec, Inc. (San Jose, USA). Chemerin Receptor 23, anti-human, monoclonal antibody (ChemR23 Ab) and isotype IgG3 were purchased from R&D Systems (Minneapolis, USA). PE-labeled anti-human CD80, monoclonal FITC-labeled anti-human CD206 Ab and the IgG1K isotype control were purchased from BD Biosciences (San Jose, California USA).

Primary cell purification and cell culture

White blood cells from healthy volunteers were isolated as described previously [25] from buffy coat (Blutspendezentrum, Zurich, Switzerland) using Histopaque-1077 (Sigma-Aldrich) gradient centrifugation. Briefly, monocytes were purified by capture with anti-CD14 Abs coupled to MACS Microbeads (MiltenyiBiotec, Bergisch Gladbach, Germany) and let differentiate into macrophages for 7 days at 37°C and 5% CO2 in RPMI 1640 (Sigma-Aldrich) supplemented with 5% Fetal Calf Serum (Bioconcept, Allschwil, Switzerland), 5% human AB serum (Sigma-Aldrich) and 1% penicillin/streptomycin (Invitrogen, Zug, Switzerland).

Human THP-1 monocytes were cultured in RPMI1640 (Sigma-Aldrich) supplemented with 10% FCS (Bioconcept), 20 mM glutamine (Invitrogen), at 37°C and 5% CO2. For differentiation into macrophages, the THP-1 cells were stimulated for 48 hours with phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich).

RNA isolation and RT reaction for real-time PCR

Total RNA from primary monocytes and macrophages was isolated using the Rneasy mini kit from Qiagen AG (Hombrechtikon, Switzerland). cDNA was prepared by RT reaction from 1µg total RNA using the Superscript III reverse transcriptase (Invitrogen).

Relative quantification of ChemR23 expression levels

ChemR23 expression levels were quantified by real-time PCR using the SYBR Green master mix kit (Roche Diagnostics, Rotkreuz, Switzerland) on the Light Cycler 480 (Roche

Diagnostics). The reactions were performed under following conditions: preheating 10 min at 95°C followed by 45 cycles of denaturation 5 sec at 95°C, annealing 10 sec at 60°C and extension 6 sec at 72°C. Relative gene expression was normalized to GAPHD. Primers are listed in the Supplementary table 2.1. Data was analyzed with Light Cycler 480 software (Roche Diagnostics).

Rapid amplification of cDNA ends

Total RNA was extracted from primary monocytes and macrophages as described above. 5´rapid amplification of cDNA ends (RACE) was performed using the RLM-RACE Kit (Ambion, Rotkreuz, Switzerland) according to the manufacturer's instructions. 3 nested PCR reactions were performed using the kit's outer and inner adaptor forward primers and ChemR23 specific reverse primers. Primer sequences are listed in Supplementary table 2.2. The PCR products were separated on a 2% agarose gel and the corresponding bands were extracted from the gel and sequenced.

Analysis of splicing variants

Novel exons detected by the RACE were verified by PCR using exon 1, 3 and 4 specific primers in combination with the RACE inner reverse primers. All primer sequences are listed in the Supplementary table 2.3. PCR products were separated on 2% agarose gel by electrophoresis, extracted from the gel and sequenced.

Generation of promoter constructs

Promoter constructs were amplified by PCR with primers containing the XhoI, and HindIII restriction sites – in forward and reverse primers, respectively (for sequence see Supplementary table 2.4). PCR products were TA subcloned into the pCRII vector (Invitrogen) and the promoter constructs were cut out using XhoI and HindIII restriction enzymes from Fermentas GmbH (St. Leon-Rot, Deutschland). Constructs were further subcloned into the promoterless vector pGL4.17 from (Promega, Madison, WI , USA) upstream of the firefly luciferase gene. All constructs were sequenced.

Transcription assays

10 µg construct, and 0.25 µg internal control phRL-SV40 (Promega) were transfected by electroporation into 6x106 THP-1 cells. Electroporation was done under the following conditions: 200 V, 950 µF capacitance, and ∞ resistance. After electroporation cells were seeded in RPMI 1640 with 10% FCS and 20 mM glutamine. After 2 h, cells were stimulated

with PMA for 48 house to differentiate into macrophages. THP-1 macrophages were then washed with PBS, lysed in 500 µl passive lysis buffer, and 20 µl of the lysate was used for dual luciferase reporter assays (Promega). The luciferase and renilla activities were measured on the luminometer Lumat LB-9507 (Berthold Technologies, Regensdorf, Switzerland). All experiments were done three times in triplicate.

FACS analysis

APC-labeled anti-human ChemR23Ab with the isotype control IgG3, and a monoclonal PE-labeled anti-human CD80 and a monoclonal FITC-labeled anti-human CD206 with the IgG1K isotype control were used for the FACS analysis. Briefly, cells were resuspended in PBS containing 2.5% FCS, and after addition of 10 µl Ab or 4 µl isotype control incubated in the dark for 30 min at 4°C before analysis on a FACS Calibur analyzer (BD Biosciences, San Jose, USA).

Chemotaxis assay

A total of 105 macrophages were placed on a 96-wellmembrane (5.7-mm diameter, 5-µm pore size; ChemoTX from NeuroProbe, Gaithersburg, MD) in RPMI 1640 containing 0.1% BSA (Sigma-Aldrich). The cells were allowed to migrate toward 10 nM chemerin for 60 min. Migrated cells were fixed (2% paraformaldehyde) and stained with DAPI (Sigma-Aldrich), and migration was quantified as the total pixel count of DAPI-stained nuclei under the fluorescence microscope (two photos per membrane and three replicate wells per treatment). Migration indices were calculated over control values.

IL-10 ELISA

IL-10 concentrations in supernatants of human macrophages were measured using commercially available human IL-10 ELISA (BioLegend, San Diego, USA) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed with Graph Pad Prism 4.03. The levels of ChemR23 mRNA and the activities of the different ChemR23 promoters in the luciferase assays were compared using a two-sided t-test. A two-sided $p < 0.05$ was considered significant.

2.1.4 Results

ChemR23 expression is upregulated during monocyte differentiation to macrophages, and regulated by cytokines and TLR ligands in macrophages

Human primary monocytes transcribe and express ChemR23 on their surface (Figure 2.1A and B). We quantified ChemR23 mRNA levels during 7 days differentiation of monocytes to macrophages. ChemR23 mRNA levels gradually increased from day 1 to day 3 and then remained on the same high level (Figure 2.1A). ChemR23 protein expression also significantly increased after monocyte differentiation to macrophages. (Figure 2.1B and C).

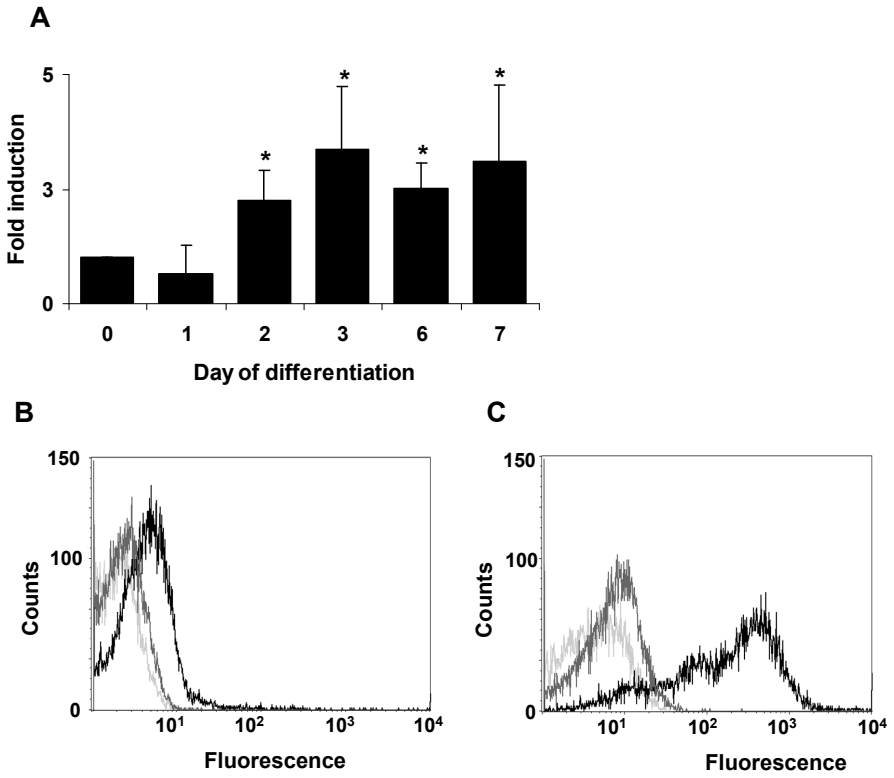

Figure 2.1: ChemR23 expression on monocytes and macrophages

A. Relative mRNA expression of ChemR23 during the 7 days differentiation of monocytes to macrophages. The values were normalized for GAPDH mRNA. Bars indicate the mean of 3 independent experiments as fold induction of control. *p<0.05, **p<0.01. B. ChemR23 protein expression on monocytes C. ChemR23 protein expression on macrophages. Autofluorescence of the cells is shown in light grey, the isotype control in grey and cells labeled with ChemR23 Ab in black. Representative graphs of at least 3 independent experiments are shown.

To asses the expression of ChemR23 in macrophages after activation, we stimulated macrophages with cytokines and TLR ligands. ChemR23 transcription and protein expression was downregulated by IL-13 and IL-4 (Figure 2.2A, B and C) - cytokines leading to the M2 phenotype, and by stimulation with the synthetic TLR8 ligand 3M002. On the other hand,

ChemR23 transcription was upregulated by the TLR-4 ligand LPS and by the proinflammatory IFN-γ - stimuli responsible for induction of the M1 phenotype. LPS stimulation also increased ChemR23 protein expression (Figure 2.2D), while IFN-γ and 3M002 did not significantly change the protein levels (data not shown). Stimulation with IL-3, IL-8, MCP1, TNFα, TGFβ, and IL-6 and the TLR ligands CpG, 3M001 and poly I:C did not significantly affect ChemR23 transcription (Figure 2.2A). In a chemotaxis assay we show that these changes on the mRNA and protein level are reflected in functionality. Naive (unstimulated) as well as LPS stimulated M1 macrophages actively migrate toward chemerin. However, IL-4 and IL-13 stimulated M2 macrophages only show unspecific migration and are not attracted by chemerin (Figure 2.2E). These results indicate that alternatively activated M2 macrophages do not express functional ChemR23 and thus cannot migrate towards chemerin.

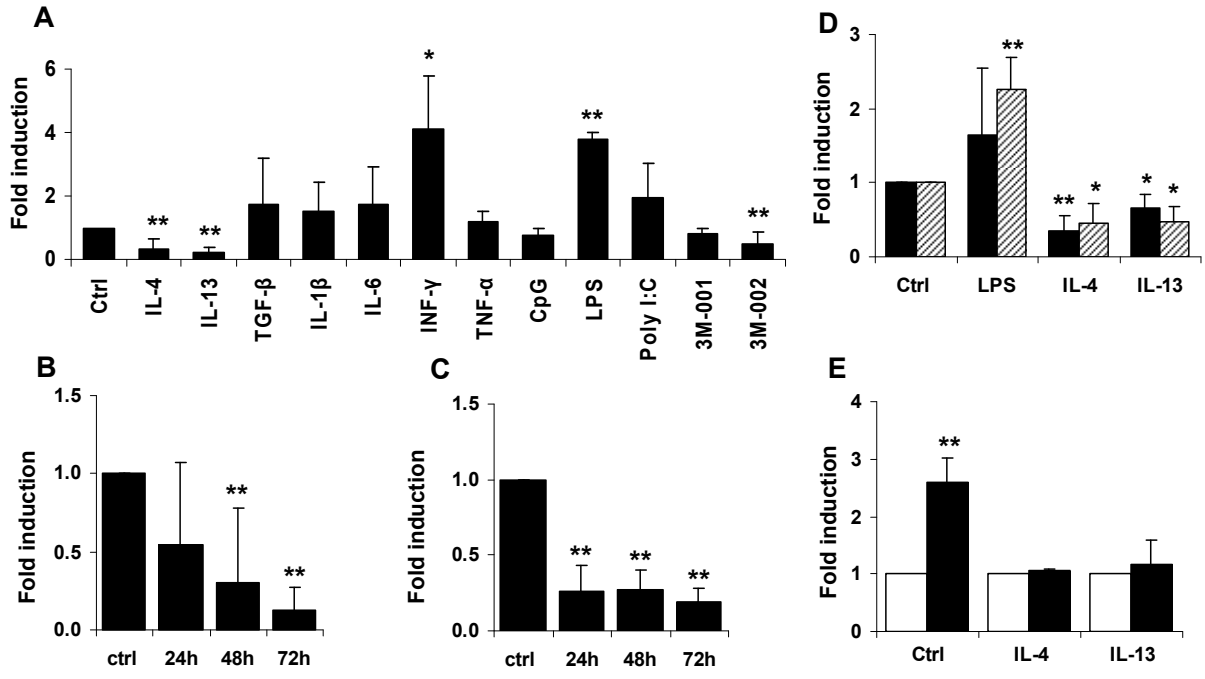

Figure 2.2: ChemR23 expression and function in macrophages in response to inflammatory and anti-inflammatory stimuli

A. Relative mRNA expression of ChemR23 after stimulation of human macrophages with different stimuli for 24 hours (IL-4 10ng/ml, IL-13 10ng/ml, TGF-β 1ng/ml, IL-1β 1ng/ml, IL-6 10ng/ml, IFN-γ 50ng/ml, TNF-α 1ng/ml, CpG 100ng/ml, LPS 100ng/ml, Poly I:C 1ng/ml, 3M001 3μM, 3M002 3μM). ChemR23 mRNA expression in macrophages stimulated for 24, 48 and 72 hours with B. IL-4 and C. IL-13. All values were normalized for GAPDH and are presented relative to unstimulated macrophages (ctrl 24h). Bar indicates the means of 3 independent experiments. D. ChemR23 protein expression on stimulated macrophages as measured by FACS. Values are presented relative to unstimulated macrophages. Bar indicates the means of 3 independent experiments. In black: 48 hours stimulation, dashed: 72 hours stimulation. E. Chemerin chemotaxis assay. Unstimulated, IL-4 and IL-13 stimulated macrophages were allowed to migrate toward 10nM chemerin. Bars show the mean migration index of 3 independent experiments. Migration of each set is shown relative to basal migration without the use of chemerin. *$p<0.05$, **$p<0.01$.

ChemR23 is transcribed from 3 transcription start sites, and is differentially spliced in human monocytes and macrophages

We used the 5′ RACE to detect ChemR23 transcription start sites in human primary monocytes and macrophages. We identified 3 transcription starts in both monocytes and macrophages (Figure 2.3A and B). The first start site lies upstream of exon 1 in a region homologous to the promoter region and to the start site previously described in murine microglial cells [26]. The other two start sites are located in previously unreported genomic regions 12 950 and 21 370 base pairs (bp) downstream from P1, respectively.

To analyze the functionality of the putative promoter regions upstream of the identified transcription starts, fragments of 500 bp and 1000 bp for each promoter were cloned upstream of the firefly luciferase gene, and the luciferase activity was measured in transfected THP1 macrophages. The promoters upstream of exon 1 (P1) and 4 (P3) increased transcription of the reporter gene, while the promoter upstream of exon 3 (P2) showed only basal activity similar to the promoterless control vector, suggesting it may only be essential in other cell types (Figure 2.4A).

In addition, the RACE experiment indicated that several ChemR23 mRNA isoforms are transcribed from promoter P1 located upstream of exon 1. To characterize these splice forms, we performed several RT-PCR reactions with primers specific for each exon. We identified 3 splice variants in macrophages and 4 in monocytes for mRNAs starting with exon 1 (transcribed from P1), and we confirmed the presence of ChemR23 mRNAs starting with exon 3 (transcribed from P2) and exon 4 (transcribed from P3) (Figure 2.3B) with no differential splicing.

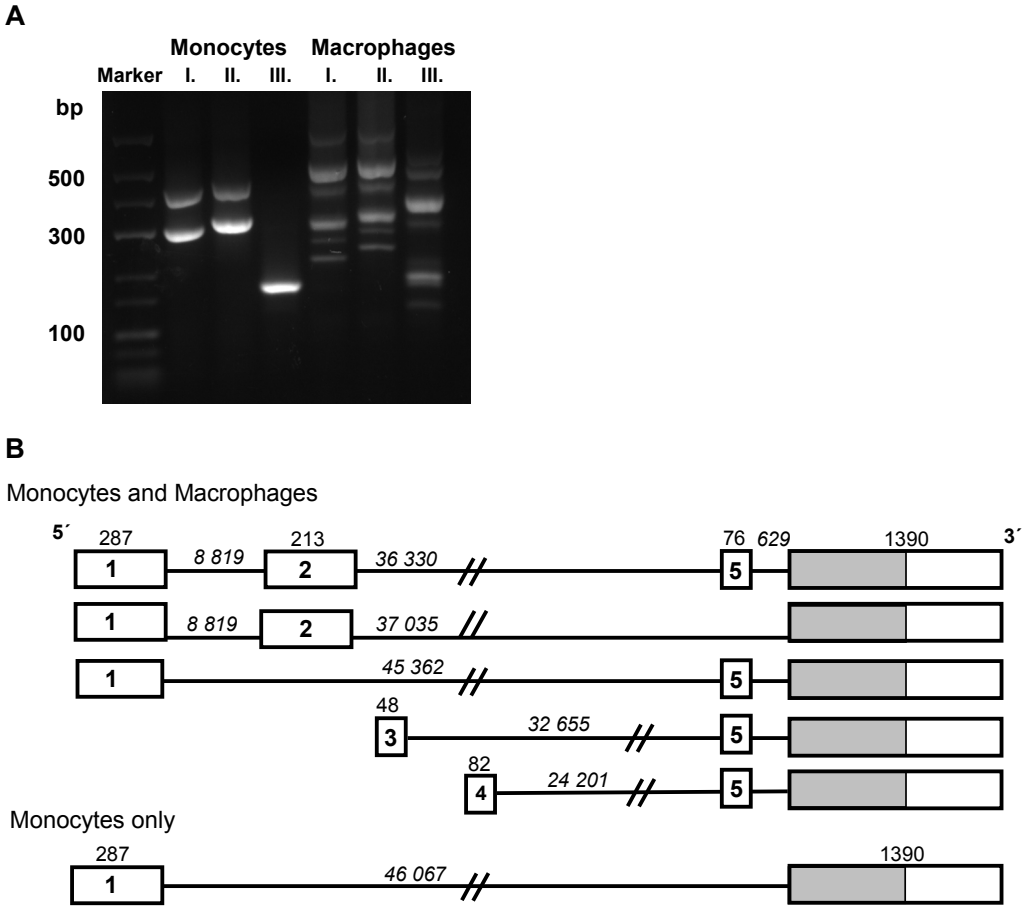

Figure 2.3: ChemR23 transcription start sites and mRNA isoforms in monocytes and macrophages

A. Nested RT-PCR products after RACE visualized on a 2% agarose gel. Race specific nested amplification using in combination with 5'RACE adapter nested inner primer the following gene specific primers: I. coding region specific reverse primer I., II. coding region specific reverse primer II, III. exon 5 specific reverse primer. B. Schematic of all differently spliced products identified by RACE and RT-PCR in monocytes and macrophages. Boxes represent exons and lines introns. The grey boxes represent the ChemR23 coding region. Numbers represent lengths of exons and introns in bp. ChemR23 gene is located on chromosome 12, contig 12q24.1. Intercontig position of transcription start sites: start site in exon 1: 108733094, start site in exon 2: 108720144, start site in exon 3: 108711724.

In M1 macrophages, ChemR23 is preferentially transcribed from promoter P3

To asses, which of the indentified promoters drives the increased transcription of ChemR23 in M1 macrophages, we quantified all mRNA variants transcribed from P1 and P3 in unpolarized, LPS and IFN-γ stimulated M1 macrophages. While the mRNA transcribed from P1 remained on the same level after IFN-γ and LPS stimulation, the mRNA transcribed from P3 increased 5 to 10 fold, respectively (Figure 2.4B). To locate the regulatory elements responsible for the transcriptional activation in the promoter region P3, we performed luciferase activity assays in THP1 macrophages transfected with a plasmid containing 1000pb of promoter P3 upstream of the luciferase gene. However, no increase in luciferase activity

was observed after stimulation of these macrophages with LPS or IFN-γ (data not shown). Congruent with the lack of induction of luciferase activity by LPS and IFN-γ, *in silico* analysis of the promoter P3 using the software MatInspector from Genomatix [27] did not predict any NF-κB, AP1, IRF-3 transcription factor binding sites, or IRF-1, IRF-9 and STAT-1 binding sites necessary for TLR-4 [28] and IFN-γ signaling [29], respectively.

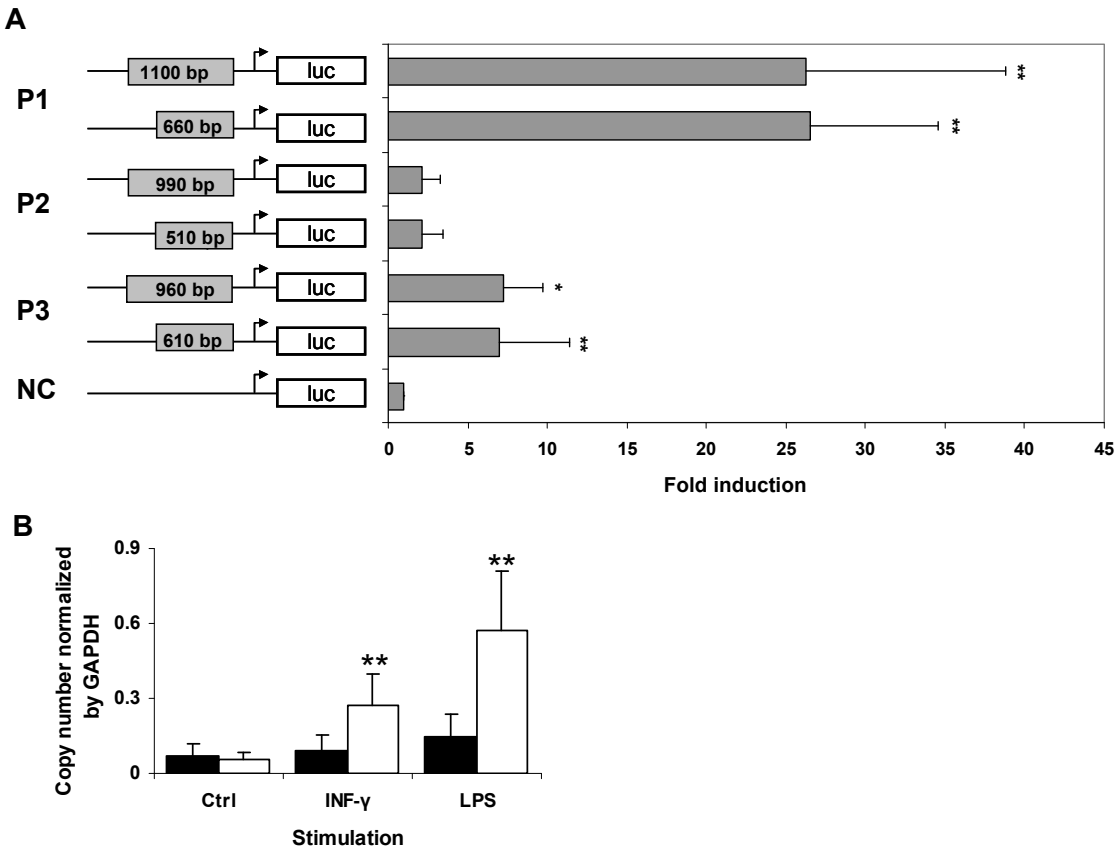

Figure 2.4: ChemR23 transcription from alternative promoters

A. Luciferase activity measured in THP-1 macrophages transfected with the luciferase gene under the control of different ChemR23 promoter fragments. P1 - promoter upstream of exon 1, P2 - promoter upstream of exon 3, P3 - promoter upstream of exon 4. Bars represent the fold induction relative to the promoterless vector pGL4.18, which was used as a negative control (NC). All experiments were done 3 times in triplicate. Firefly luciferase activity was normalized by the Renilla luciferase activity. B. Absolute quantification of ChemR23 mRNA variants transcribed from P1 (black) and P3 (white). ChemR23 mRNA was quantified in unstimulated and IFN-γ and LPS stimulated primary human macrophages (24h). Values were normalized to GAPDH mRNA copy numbers. Bars indicate the mean of 3 independent experiments as fold induction of control. *p<0.05, **p<0.01.

RvE1 signaling in M1 macrophages

Our results show that resting and M1 macrophages express functional ChemR23 on their surface while M2 macrophages do not. We therefore surmised that the pro-resolving actions of RvE1 must be mediated through ChemR23 on resting or inflammatory macrophages. To investigate whether RvE1 causes a re-polarization of inflammatory M1 macrophages to an

M2 like phenotype, we sequentially stimulated primary human macrophages with LPS followed by either RvE1, IL-4 (control stimulus for M2 polarization), or medium without stimulus, and characterized the polarization phenotype of the macrophages. As expected, LPS stimulated M1 macrophages exhibited an increased transcription of IL-1β, TNFα and cell surface expression of CD80 [25,30]. Upon removal of the stimulus, IL-1β, TNFα and ChemR23 mRNA and CD80 protein levels returned to levels comparable to untreated macrophages. (Figure 2.5 and Figure 2.6A). Stimulation of these M1 macrophages with RvE1 lead to a similar reversal of IL-1β, TNFα and ChemR23 transcription. In contrast, RvE1 increased IL-10 transcription but not secretion, while RvE1 and IL-4 lead to a small but not significant increase in CD80 expression (Figure 2.6). In addition, only IL-4 increased CD206 expression in M1 macrophages. These data indicate that RvE1 may have the potential to re-polarize M1 macrophages toward an anti-inflammatory phenotype different from the M2 polarization triggered by IL-4.

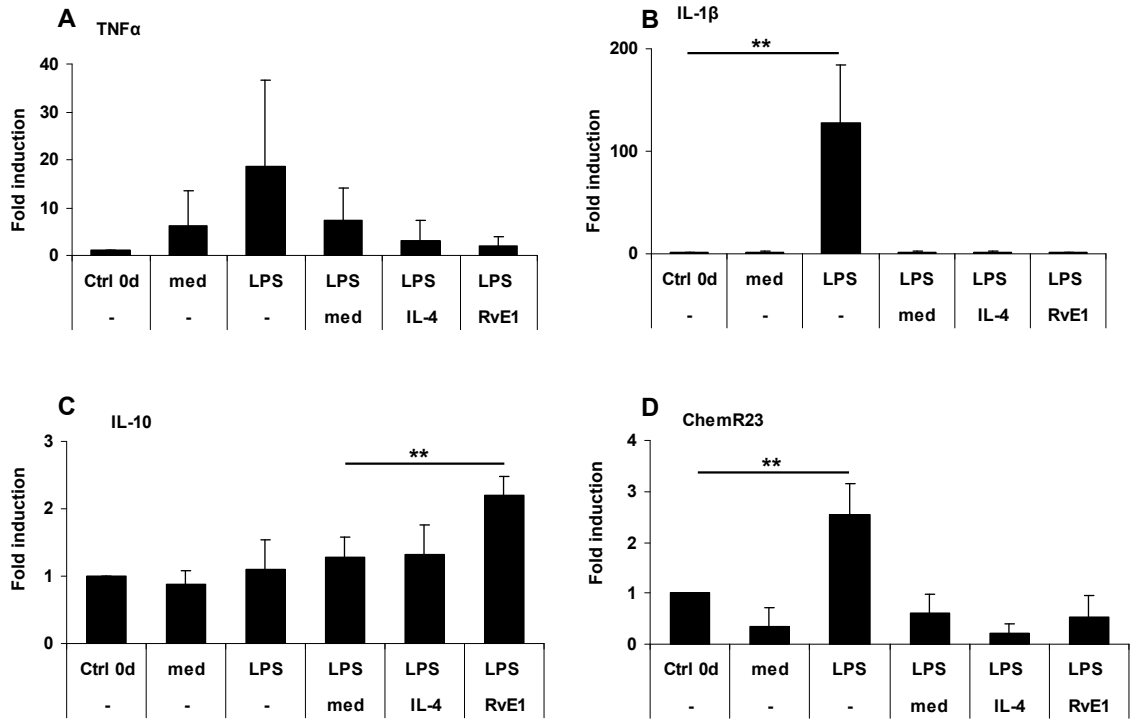

Figure 2.5: Quantification of cytokine and ChemR23 mRNA levels following re-stimulation of LPS activated primary human M1 macrophages with RvE1

A. TNFα . B. IL-1β. C. IL-10 D. ChemR23. Upper line – initial 3 day stimulation, bottom line – re-stimulation. Ctrl 0d – unstimulated macrophages day 0, med – medium with no stimulus. All values were normalized for GAPDH and are presented relative to unstimulated macrophages day 0. Bars indicate the mean of 3 independent experiments as fold induction of control. *p<0.05, **p<0.01.

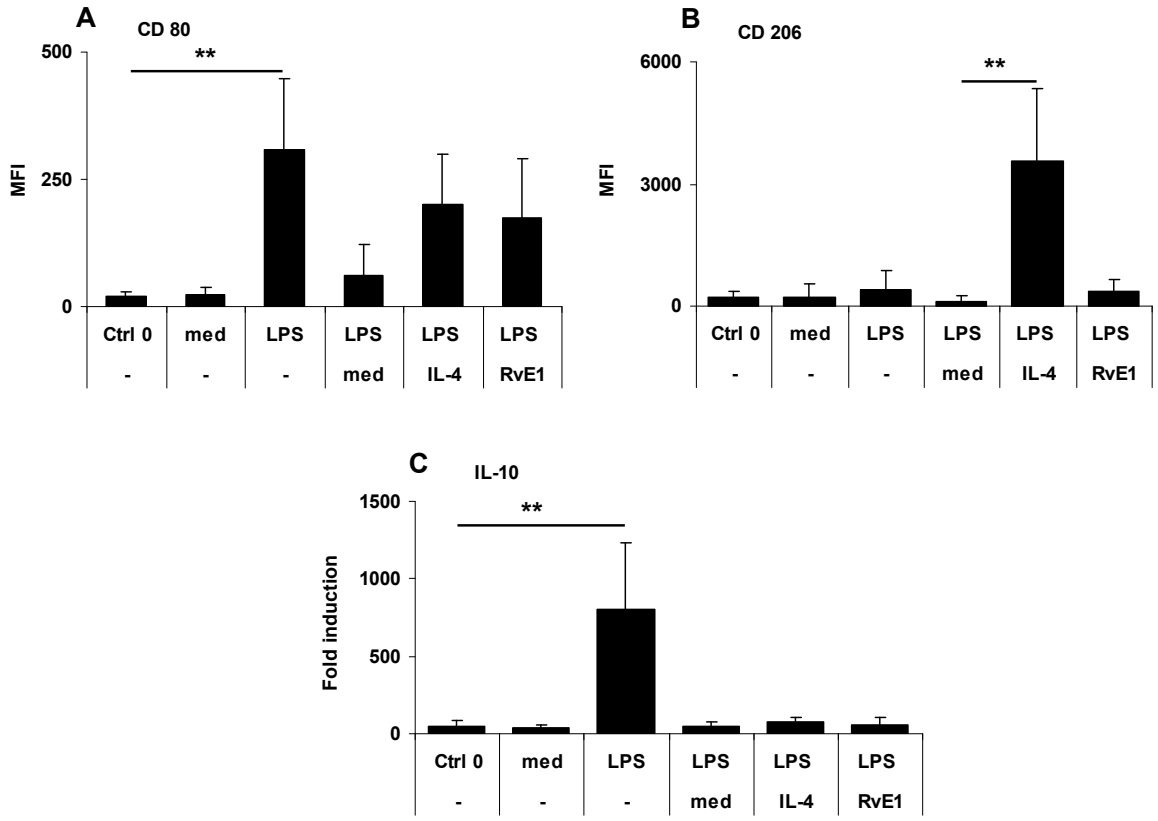

Figure 2.6: Cell surface expression of CD80 and CD206 and secretion of IL-10 following re-stimulation of LPS activated primary human M1 macrophages with RvE1

A. CD80 protein expression measured by FACS. B. CD 206 (mannose receptor) protein expression measured by FACS. C. IL-10 secretion measured by ELISA in supernatants of macrophages. Values are presented relative to unstimulated macrophages. Upper line – initial 3 day stimulation, bottom line – re-stimulation. Ctrl 0d – unstimulated macrophages day 0, med – medium with no stimulus. Bar indicates the means of 3 independent experiments. *$p<0.05$, **$p<0.01$.

2.1.5 Discussion

Classically activated M1 macrophages secreting high levels of inflammatory cytokines are implicated in the initiation of and in sustaining inflammation [31], while alternatively activated M2 macrophages have anti-inflammatory properties and are associated with wound healing and tissue repair [3]. As a receptor for the chemoattractant chemerin and the lipid mediator RvE1, ChemR23 plays a role in immigration of macrophages to the inflamed area [12] as well as in the resolution of inflammation [13,23]. However, little is known about its regulation during different stages of inflammation or in different activation states of macrophages. Here we show that ChemR23 transcription and protein expression increase during differentiation of monocytes to macrophages and are further amplified in classically activated M1 macrophages *in vitro*. M1 macrophages migrate towards the ChemR23 ligand chemerin and increase IL-10 transcription in response to RvE1 stimulation, suggesting that RvE1 may re-polarize human M1 macrophages to an intermediate resolution phase phenotype. In contrast, ChemR23 expression is not detectable in alternatively activated M2 macrophages, which are also not responsive to ChemR23 signaling.

The regulation of ChemR23 expression was previously studied in mouse macrophages, where ChemR23 was down-regulated by inflammatory stimuli such as TLR ligands (LPS, CpG) and inflammatory cytokines, and upregulated by TGFβ [32,33]. The authors of these studies concluded that ChemR23 has a role in naive and anti-inflammatory macrophages only. In contrast, we and others [13] show that ChemR23 mRNA levels increase in human monocytes and macrophages after stimulation with LPS or the inflammatory cytokines TNF-α and IFN-γ, suggesting that there are differences between mice and men in the regulation and eventually function of ChemR23. This notion would be supported by the fact that chemerin has ChemR23 dependent anti-inflammatory and protective effects in some mouse models of inflammation [34,35], while in humans increased chemerin concentrations are associated with pathologies connected with chronic and systemic inflammation [36,37,38,39] and chemerin was shown to induce inflammatory signaling in chondrocytes [40]. The differential expression pattern of ChemR23 on stimulated macrophages and the possible opposite effect of chemerin on inflammation between mice and men may indicate that the role of ChemR23 signaling differs between the two species.

We report the identification of 3 ChemR23 transcription start sites in human primary monocytes and macrophages and show that ChemR23 is differentially spliced. Basal transcription in human monocytes and naive macrophages is driven equally from promoters

P1 and P3 and transcripts from promoter P1 are differentially spliced leading to 4 splicing variants in monocytes and 3 in macrophages. All differentially spliced exons are non-coding and have no effect on the final size and sequence of the protein. However, such differential splicing and the presence of various mRNA isoforms may represent an additional way of ChemR23 regulation, as sequence characteristics within the 5' UTR play an important role in the differential regulation of translation efficiency and mRNA stability [41].

From the three identified promoters only P1 and P3 are used in monocytes and macrophages, while transcription from promoter P2 is basically absent. Similarly, two distinct start sites and several differentially spliced isoforms were reported for the mouse homologue of ChemR23 (DEZ) in mouse neuroblastoma and microglia cells [26,42]. Such usage of alternative promoters has been previously described to enable differential transcriptional regulation in different cell types or developmental stages, or upon stimulation [43]. Recent evidence suggests that more than 50% of human protein coding genes have multiple alternative promoters [44]. Promoter P2 is therefore likely to be used in other cells types than monocytes and macrophages.

Stimulation of primary human macrophages with LPS or IFNy increased transcription downstream of promoter P3, indicating a preferential use of this promoter in M1 macrophages. However, we did not detect transcription factor binding sites necessary for TLR-4 [28] or IFN-γ signaling [29] within 1000 bp upstream of the transcriptional start site, neither by transcriptional activity experiments using the reporter constructs nor by *in silico* analysis. These results indicate that the regulatory elements necessary for TLR-4 and IFN-γ signaling are located either further upstream or downstream of the transcription start site. This would be in agreement with the findings that transcriptional enhancer elements can be located in the first intron [45,46] or even several kb from the transcription start site [47].

Our results show that only naive and inflammatory M1 macrophages can be responsive to ChemR23 mediated triggering, which implicates that RvE1 exerts its pro-resolving actions only on these macrophage phenotypes. In an effort to assess whether RvE1 initiates re-polarization of M1 macrophages, we measured the expression of cytokines and surface markers characteristic for M1 or M2 macrophages after re-stimulation of M1 macrophages with RvE1. Re-stimulation resulted in a mild but not significant increase in CD80 expression, similar to the typical M2 stimulus IL-4, while in contrast to IL-4, RvE1 did not induce mannose receptor expression but increased IL-10 transcription without increasing translation. Hence, re-stimulation of M1 macrophages with RvE1 may lead to sustained CD80 expression and to increased IL-10 expression, suggesting an anti-inflammatory and eventually pro-

resolving effect of RvE1 on M1 macrophages. However, this pro-resolving effect does not result in an IL-4 mediated M2 polarizing effect but rather leads to an intermediate macrophage phenotype.

Intermediate macrophage phenotypes have recently been indentified during the resolution phase of inflammation *in vivo*. Bystrom et. al. isolated resolution phase macrophages which secreted high levels of anti-inflammatory IL-10 but little inflammatory cytokines and which expressed the mannose receptor (properties of M2 macrophages) as well as high levels of COX-2 (a property of M1) [7]. A transcriptomic analysis showed that resolution phase macrophages transcribe high levels of IL-10, COX-2 and of the mannose receptor, and despite the low secretion, also high levels of inflammatory cytokines [48]. In the same mouse model, peritoneal injection of RvE1 induced the formation of $CD11b^{low}$ macrophages with high phagocytic capacity, reduced TNFα and increased IL-10 secretion supporting the pro-resolution effect of RvE1 in mouse macrophages [49]. Although the characteristics of the resolution phase mouse macrophages described in the above mentioned studies differ from the characteristics of the RvE1 re-stimulated M1 human macrophages in our experiments, increased IL-10 expression seems to be a common denominator of such intermediate pro-resolving macrophages. In agreement with these data, we show that RvE1 induced IL-10 transcription in primary human M1 macrophages suggesting that RvE1 may re-polarize human primary M1 macrophages towards a resolution phase macrophage.

Unexpectedly, we observed no increase of IL-10 secretion upon re-stimulation of M1 macrophages with RvE1, arguing for post-transcriptional regulation of IL-10 secretion. Indeed, IL-10 secretion was shown to be regulated by microRNAs [50] and AU-rich elements present in the 3′-UTR of the IL-10 mRNA [51], which may explain the low IL-10 secretion observed in RvE1 re-stimulated M1 macrophages despite increased transcription rates. Alternatively, differences in the experimental set-up may lead to the not congruent results between the *in vivo* mouse studies and our *in vitro* human studies. Following systemic application of RvE1 in mice multiple cell types are affected such as endothelial cells through ChemR23 [11,13] and neutrophils through the BLT1 receptor [52], which in turn may stimulate macrophages. In contrast, these additional stimuli lack in our *in vitro* study on primary human macrophages.

In summary, we show that ChemR23 is tightly regulated in response to inflammatory and anti-inflammatory stimuli. The high expression of ChemR23 in naive and M1 macrophages supports the role of ChemR23 in the attraction of macrophages to inflamed tissue by chemerin and in the initiation of resolution of inflammation through RvE1 signaling in human

macrophages. In contrast, the role of ChemR23 mediated signaling is limited in M2 macrophages.

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2.1.7 Supplementary data

Supplementary table 2.1: Primers for quantitative PCR, 5´- 3´

Gene	Forward	Reverse
ChemR23 all RNA variants	TTGGCTGAGGACTCACATTG	CTGATCTTGCACATGGCTGT
ChemR23 variants transcribed from P1	TCAGAGGGGGATCTTGAATG	GGGGATAAGTCCTCCAAAACCA
ChemR23 variants transcribed from P3	AAACTCTGGAAGGAGACATGAGGAACT	GGGGATAAGTCCTCCAAAACCA
GAPDH	CCCATGTTCGTCATGGGTGT	TGGTCATGAGTCCTTCCACGATA
Human IL-1β	TACCTGTCCTGCGTGTTGAA	TCTTTGGGTAATTTTTGGGATCT
Human TNFα	GAGTGACAAGCCTGTAGCCCATGTT GTAGCA	GGCAATGATGATCCCAAAGTAGAC CTGCCCAGACT
Human IL-10	GATCCAGTTTTACCTGGAGGAG	CCTGAGGGTCTTCAGGTTCTC

Supplementary table 2.2: Primers for RACE, 5´- 3´

	Forward	Reverse
ChemR23 inner primers	GCTGATGGCGATGAATGAACACTG	I. GGGGATAAGTCCTCCAAAACCA II. TCCTGGTCACCCTGGCTTCC III. AGTCCTCAGCCAATCAGTCCCTGT
ChemR23 outer primers	GAACACTGCGTTTGCTGGCTTTGATG	AATCCCGAGGAAGCAGACGAT

Supplementary table 2.3: Primers for differential splicing, 5´- 3´

Exon	Forward	Reverse
Exon 1	TCAGAGGGGGATCTTGAATG	-
Exon 2	TGGTCACAGCAACTCACCAT	AGATGCCATCAGCTTCTGCT
Exon 3	AGTGCTCAGGGTGGGGGACA	-
Exon 4	AAACTCTGGAAGGAGACATGA	-
Exon 5	-	AGTCCTCAGCCAATCAGTCCCTGT
Coding exon	-	I. GGGGATAAGTCCTCCAAAACCA II. TCCTGGTCACCCTGGCTTCC

Supplementary table 2.4: Primers for promoter subcloning, 5´- 3´

Promoter	Forward 1000 bp	Forward 500 bp	Reverse
P1	TACGCTCGAGTGGTGGTTCA CACGGCTTAC	GAGACTCGAGTGATGGGGTG ACCCTGAG	GAAGAAGCTTCATTCAAGA TCCCCCTCTG
P2	CTGGCTCGAGTCTCAAACTC CTGGCTTCAAG	GAGTCTCGAGTGCTTTCTGA AATGCGATTG	GAGTAAGCTTGTCCACAGC ACCTGAATGT
P3	TAGACTCGAGTTGGGGCCAC CAAAGGCACC	CAGACTCGAGAACAGCCAG TACAGCTAGAAT	TGTAAAGCTTCCACCTCCCT ATGTTAGTTC

2.2 Low dose aspirin is associated with plasma chemerin levels and may reduce adipose tissue inflammation

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2.2.1 Abstract

Chemerin is a peptide chemoattractant for macrophages and an adipokine regulating adipocyte differentiation and metabolism. Plasma chemerin is increased in chronic inflammatory diseases and in obesity. As inflammation and obesity are risk factors for coronary artery disease (CAD), we investigated possible associations of plasma chemerin with inflammatory markers and atherosclerosis in a CAD case-control study (n=470). Chemerin levels were associated with C-reactive protein, BMI and LDL levels, and negatively associated with HDL levels. Mean plasma chemerin levels were similar in controls and CAD patients but significantly higher in CAD patients not taking low dose aspirin. To investigate the mechanism of chemerin reduction by aspirin, we analyzed chemerin expression in hepatocytes and adipocytes treated with aspirin in the presence and absence of inflammatory cytokines. Chemerin expression was upregulated by pro-inflammatory stimuli in adipocytes but not in hepatocytes. Treatment of stimulated hepatocytes and adipocytes with aspirin did not affect chemerin expression. However, treatment of inflammatory M1 macrophages with aspirin reduced secretion of the pro-inflammatory cytokines IL-1β and IL-6, and increased secretion of the anti-inflammatory IL-10. In summary, we show that plasma chemerin levels are associated with markers of inflammation and that they are significantly higher in CAD patients not treated with low dose aspirin. In addition, we show that low dose aspirin treatment reduces pro-inflammatory cytokine secretion by macrophages, which may lead to reduced chemerin secretion by adipocytes and may be a reason for the lower chemerin levels in the circulation of CAD patients on low dose aspirin.

2.2.2 Introduction

Cardiovascular disease is the leading cause of morbidity and mortality in developed countries and an emerging health problem worldwide. Coronary artery disease (CAD) is one of the most common forms of cardiovascular disease caused by progressive atherosclerotic lesion formation leading to narrowing of the arterial lumen [1].

The major independent risk factors for CAD are high blood pressure, elevated serum total and low-density lipoprotein cholesterol (LDL), low serum high-density lipoprotein cholesterol (HDL), diabetes mellitus, advanced age, and smoking [2]. Recently, central obesity and the metabolic syndrome associated with obesity have been identified as additional risk factors. The adipose tissue is today recognized as an important player in obesity-mediated cardiovascular disease [3] not only through its role in energy storage but also due to its role as an active endocrine organ [4]. Disrupted lipid and glucose metabolism in obese adipose tissue and low systemic inflammation accompanying obesity accelerate the development of cardiovascular disease [5].

Chemerin is an adipokine and a chemokine with a role in inflammation. It is secreted as a non-active precursor protein, which can be activated through cleavage of its C-terminus by proteases of the coagulation and fibrinolytic cascades, and proteases released from activated neutrophils or mature adipocytes [6,7]. Chemerin is highly expressed by adipocytes and after activation triggers the maturation of preadipocytes into adipocytes [8,9]. This role in adipocyte maturation is reflected by the positive correlation of chemerin plasma levels with the waist hip ratio and BMI [10,11,12], and by the decrease of plasma chemerin levels after bariatric surgery [13] in parallel to the decrease of body fat mass. In addition, chemerin has been detected in high amounts in inflammatory fluids [14,15,16] and in atherosclerotic lesions [17]. Chemerin is a chemoattractant for leukocytes and is thought to recruit macrophages to the site of inflammation [18].

Given chemerin´s role in inflammation as a leukocyte chemoattractant and its function in adipogenesis, we hypothesized that plasma chemerin could be associated with human atherosclerosis. We therefore investigated the association of chemerin plasma levels with markers of atherosclerosis and with CAD in a human case-control study.

2.2.3 Material and Methods

Case-control study

A total of 323 men and 147 women from Zurich volunteered to participate in the study [19]. They all signed an informed consent form. The study was approved by the local Ethics Committee and complies with the Declaration of Helsinki. The case group consisted of 249 consecutive Caucasian patients with angiographically documented CAD with more than 50% stenosis in at least 1 coronary artery. The control group consisted of 221 Caucasians with angiographically negative results and individuals with no history of CAD, stroke, or peripheral vascular disease recruited from the general population. Risk factors and the use of medication were assessed by a questionnaire. The clinical chemistry analyses were carried out on a Roche-Hitachi Modular Clinical Chemistry analyzer using commercial tests from Roche Diagnostics (Rotkreuz, Switzerland).

Power calculation

The study has a power of 80% to detect a mean difference in chemerin plasma levels of ± 1.18 ng/ml between cases and controls.

Sample preparation and ELISA

Plasma samples were collected consecutively and stored at – 20°C before analysis. Plasma was diluted 200x and total chemerin concentration was assessed with a commercially available human chemerin ELISA (R&D Systems) according to the manufacturer's instructions. The antibodies used for the ELISA are raised against AAs 21 to 157 of human chemerin. Prochemerin as well as active forms of chemerin are therefore detected by the assay.

Chemerin concentrations in 3T3-L1mouse adipocyte and Hep3B hepatocyte supernatants were measured using commercially available mouse and human chemerin ELISAs (R&D Systems) according to the manufacturer's instructions.

Cytokine concentrations in macrophage supernatants were measured with the Bio-Plex Pro multiplex ELISA assay (Bio-Rad, Cressier, Switzerland). Fluorescence signals were detected using the Luminex® 200™ System (Bio-Rad, Cressier, Switzerland). Median Fluorescence Intensity (MFI) as well as the concentration (pg/ml) was calculated by using the Bio-PlexManager™software provided by the manufacturer.

Cell culture experiments with cell lines

Human Hep3B hepatocytes were grown in RPMI-1640 (Sigma-Aldrich) medium supplemented with 10% FBS (Sigma Aldrich), 2% L-glutamine (Invitrogen) and 1% Penicillin/Streptomycin (Sigma Aldrich) at 37°C and 5% CO_2.

Mouse 3T3-L1 preadipocytes were cultured in DMEM (Sigma Aldrich) supplemented with 10% FBS and 1% Penicillin/Streptomycin. 2d after confluence, differentiation was initiated by addition of 1 µg/ml insulin (Sigma Aldrich), 115 µg/ml 1-methyl-3-isobutylxanthine (IBMX; Sigma Aldrich) and 1 µM dexamethasone (Sigma Aldrich) for 2 days. Then the medium was changed to DMEM, 10% FBS, 1% Penicillin/Streptomycin and 1 µg/ml insulin for 2 days. Thereafter, the cells were maintained in the preadipocyte medium.

Cell culture experiments with primary human macrophages

Human leukocytes were isolated from buffy coat (Blutspendezentrum Zurich, Switzerland) with Histopaque-1077 gradient (Sigma Aldrich). Peripheral blood monocytes were purified by capturing with anti-CD14 antibodies coupled to MACS Micro beads (Miltenyi Biotec, Bergisch Glad, Switzerland) according to the manufacturer's instructions. Monocytes were allowed to differentiate into macrophages for 7 days at 37°C in RPMI-1640 supplemented with 5% FBS, 5% human AB serum (Sigma Aldrich), 1% Penicillin/Streptomycin and 5% CO_2.

Relative quantification of gene expression

RNA was isolated using the RNeasy mini kit (Qiagen, Canada). cDNA was prepared by reverse transcription from 1µg total RNA using the Superscript III reverse transcriptase (Invitrogen). Chemerin and cytokine mRNA levels were quantified by real-time PCR using the SYBR Green master mix kit (Roche Diagnostics, Rotkreuz, Switzerland) on the Light Cycler 480 (Roche Diagnostics). The reactions were performed under the following conditions: preheating 10min 95°C followed by 45 cycles of denaturation 5sec 95°C, annealing 10sec, 60°C and extension 6sec 72°C. Relative gene expression was normalized to GAPDH in human Hep3B hepatocytes and macrophages, and cyclophilin A in mouse 3T3-L1adipocytes. The primers are listed in the Supplementary table 2.7.

Statistical analysis

The statistical analysis was performed using the R software 2.12.0. Two sided t-tests were applied to investigate the association of chemerin levels with binomial variables. Uni-variate analyses of the association of chemerin with other continuous variables were carried out using

the Spearman correlation. Linear regression model was used for the multi-variate analysis of the association of chemerin with CAD risk factors. Two sided t-test and factorial ANOVA were used for the comparison of chemerin concentrations between controls and cases, and between controls and patients with and without aspirin therapy, respectively. The equality of variances of the patient and control samples, and of patients taking and not taking aspirin were tested using the F-test. Logistic regression was used to assess whether plasma chemerin levels are predictive of CAD. P-values lower than 0.05 were considered statistically significant.

2.2.4 Results

Plasma chemerin levels correlate with risk factors for CAD in healthy volunteers

The characteristics of the case-control sample are shown in Table 2.1. To determine whether chemerin levels are associated with risk factors for CAD in the general population, we investigated the association of plasma chemerin levels with these risk factors in controls only. Chemerin was positively associated with BMI ($r = 0.23$, $p < 0.0001$), cholesterol ($r = 0.24$, $p < 0.0001$), LDL ($r = 0.16$, $p = 0.017$), hypertension ($t = 2.53$, $p = 0.050$), hsCRP ($r = 0.25$, $p < 0.0001$) and negatively associated with HDL levels ($r = -0.21$, $p = 0.002$) in healthy volunteers. These associations remained significant when analyzed in cases and when analyzed in the entire case-control study. In cases not taking aspirin, the associations with LDL and hypertention were no longer significant but chemerin levels remained associated with BMI, cholesterol, high sensitivity CRP (hsCRP) and HDL (Supplementary table 2.5). No difference in plasma chemerin levels was found between sexes and there was no association of plasma chemerin levels with smoking, age or diabetes.

Table 2.1: Characteristics of controls and cases

	Controls (n=221)	Cases (n=249)	P value
Age (years)	59 (53; 66)*	63 (57; 71)	0.0001
Males (%)	57	79	<0.0001
Body-mass index (BMI)	25.8 (23.0;28.0)	26.6 (24.4;29.7)	0.0001
Obesity (BMI ≥ 30) (%)	12	18	0.0101
Hypertension (%)	28	45	0.0002
Diabetes (%)	3	20	<0.0001
Current Smokers (%)	13	16	0.0002
Ever Smoked (%)	31	58	<0.0001
Aspirin treatment (%)	NA	88	
Statin treatment (%)	NA	80	
Creatinine (μmol/l)	90.0 (82.5;98.5)	89.0 (80.0;99.0)	0.38
hsCRP (mg/l)	2.4 (0.6;2.7)	1.8 (0.9;3.8)	0.0015
Cholesterol (mmol/l)	5.9 (5.2;6.4)	5.1 (4.3;5.9)	<0.0001
HDL (mmol/l)	1.6 (1.3;1.9)	1.2 (1.1; 1.4)	<0.0001
LDL (mmol/l)	3.5 (2.8; 4.0)	3.2 (2.4; 3.8)	0.006
Chemerin (ng/ml)	164.0 (146.7;189.4)	169.6 (143.6-201.1)	0.41

*median and interquartile ranges are shown.

Plasma chemerin levels are independently associated with hsCRP, cholesterol and HDL

To investigate whether the observed associations of plasma chemerin levels with CAD risk factors are independent of each other, we used a step-wise linear regression analysis initially including all associated variables (Table 2.2). In the step-wise multiple linear regression analysis, hsCRP and cholesterol were positively associated with plasma chemerin levels while HDL levels were negatively associated with plasma chemerin levels in controls. The same parameters also remained significantly associated in a multivariate analysis across the entire case-control study after adjusting for case status and medication.

Table 2.2: Multivariate regression model to predict chemerin levels in controls

	Estimate total	**P value**
hsCRP	1.954	0.0041
Cholesterol	7.579	0.0012
HDL	-19.295	0.00023

Plasma chemerin levels are increased in CAD patients not taking aspirin

Although plasma chemerin levels were associated with several risk factors, there was no significant difference of the mean chemerin levels between CAD cases and controls ($p = 0.41$). However, the range of measured chemerin differed significantly between the two groups ($p= 0.0038$), with a larger range seen in the case group. We searched for an explanation for this broad range in chemerin plasma concentrations in cases by stratification of the cases according to drug treatment. A post-hoc analysis revealed no difference in chemerin plasma levels in CAD patients not taking statins and those on statin treatment. However, chemerin levels were significantly higher in plasma of CAD patients not undergoing aspirin treatment than in patients on aspirin treatment or in controls (cases treated vs. not treated, $p = 0.009$; controls vs. not treated cases, $p = 0.007$; ANOVA $p = 0.005$) (Figure 2.7). The difference in chemerin levels remained significant after adjusting for BMI. Characteristics of the two CAD patient groups are shown in Supplementary table 2.6.

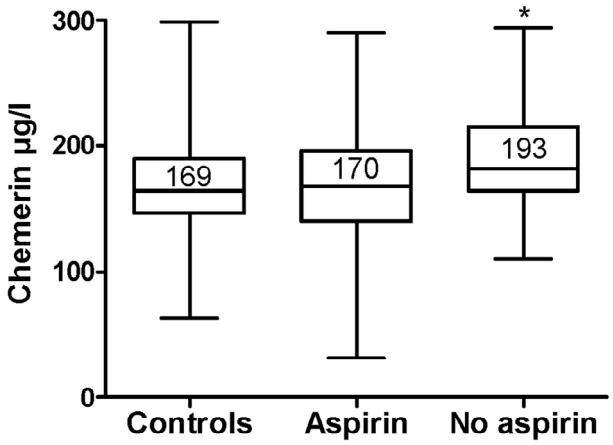

Figure 2.7: Plasma chemerin levels are higher in CAD patients not taking aspirin.

Box plot diagram of plasma chemerin levels in healthy controls (Controls), CAD patients on low dose aspirin medication (Aspirin) and CAD patients not taking aspirin (No aspirin); * $p < 0.05$

IL-1β reduces chemerin expression in hepatocytes; aspirin has no effect

Next, we sought the origin of elevated chemerin and investigated the mechanism by which aspirin reduces chemerin secretion into plasma. Chemerin is mainly expressed by the hepatocytes in the liver, and adpocytes in white adipose tissue [9,10], and chemerin expression and secretion were shown to be directly upregulated by TNFα [20,21] and IL-1β [22] in adipocytes. Liver and adipose tissue are currently believed to be the main source of plasma chemerin [23]. Therefore we postulated that these tissues are be responsible for its increase in plasma during systemic inflammation seen in obesity and CAD.

We analyzed the expression of chemerin and its regulation by inflammatory cytokines in the presence and absence of aspirin in hepatocytes using concentrations of aspirin expected in human plasma after a low dose of (100 mg/day, 2 mg/l) and high dose (400 mg/day, 20 mg/l) aspirin treatment (Produktmonographie Aspirin Cardio, Bayer AG; Arzneimittelkompedium der Schweiz). Non-stimulated hepatocytes expressed and secreted chemerin. Stimulation with IL-1β and TNFα reduced chemerin transcription, and stimulation with IL-1β also protein secretion (Figure 2.8 A and B). IL- 6 stimulation had no effect on chemerin mRNA expression and protein secretion in hepatocytes (data not shown). In addition, simultaneous treatment of hepatocytes with aspirin did not significantly change chemerin expression or secretion in control or cytokine stimulated hepatocytes (Figure 2.8 A and B). Taken together these data indicate that inflammation moderately decreases hepatocyte chemerin secretion and that aspirin treatment does not influence hepatocyte chemerin secretion.

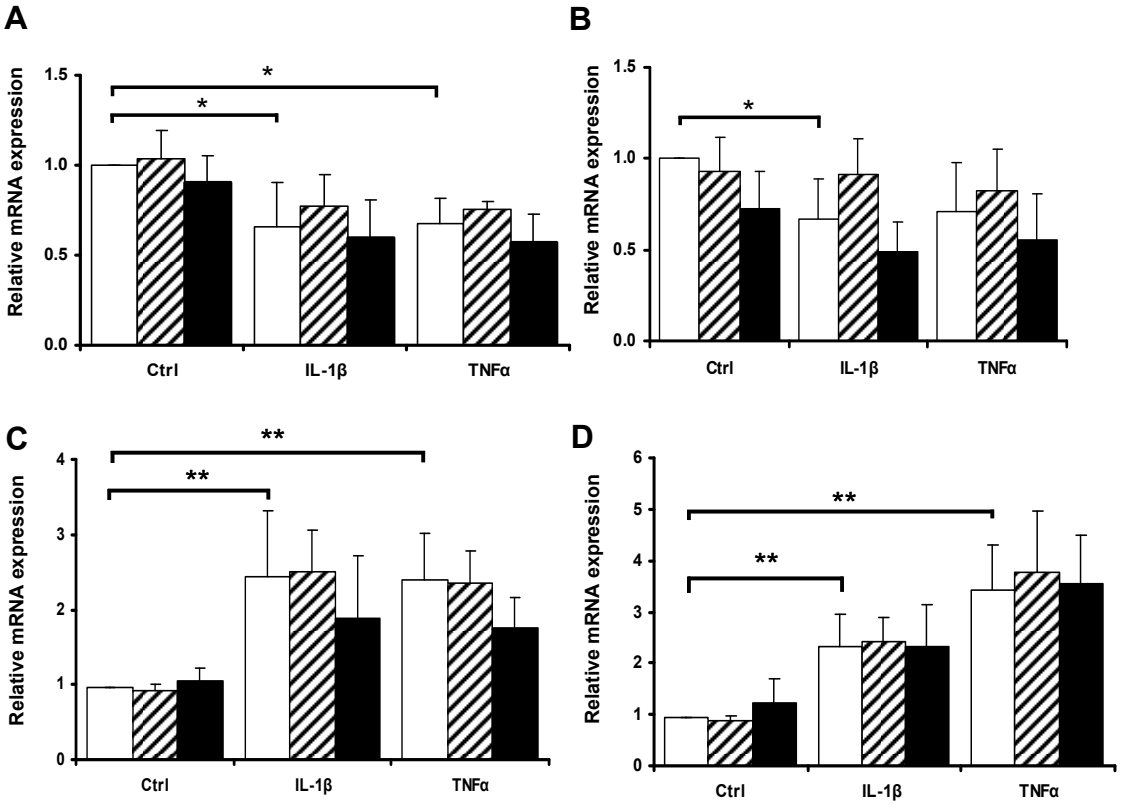

Figure 2.8: Chemerin expression and secretion in hepatocytes and adipocytes is not altered by aspirin.

Chemerin mRNA levels and secreted chemerin from Hep3B hepatocytes and 3T3-L1 adipocytes stimulated for 48 hours with 10 ng/ml IL-1β, 10 ng/ml IL-6 and 25 ng/ml TNFα in the absence and presence of aspirin. A) Chemerin mRNA in Hep3B hepatocytes. B) Chemerin protein secreted by Hep3B hepatocytes. C) Chemerin mRNA in 3T3-L1 adipocytes. D) Chemerin protein secreted by 3T3-L1 adipocytes. Indicated stimulation of the cells (white), co-stimulation of the indicated stimulation with 2 mg/l aspirin (hatched) and with 20 mg/l aspirin (black). mRNA levels were normalized to GAPDH levels and the protein levels were normalized to the control value. The chemerin concentration in the supernatant of hepatocytes and adipocytes was in the range of ng/ml. Bars represent the mean +/- standard deviation of 3 independent experiments performed in triplicates. * $p < 0.05$, ** $p < 0.01$.

Cytokines but not aspirin regulate chemerin expression in adipocytes

In adipocytes (Figure 2.8 C and D), we could confirm the previously published stimulatory effect of TNFα and IL-1β on chemerin production [20,21] [22], while no effect was observed with IL-6. However, similar to the findings in hepatocytes, aspirin co-treatment of both control and cytokine-stimulated cells did not significantly change chemerin mRNA or secreted protein levels (Figure 2.8 C and D), suggesting that aspirin does not directly regulate chemerin expression in adipocytes.

Aspirin reduces IL-1β and IL-6 expression and increases IL-10 expression in pro-inflammatory M1 macrophages

Obesity is associated with low level inflammation and with increased accumulation of mononuclear immune cells within the adipose tissue [24]. Macrophages in obese adipose tissue have in the majority the M1 phenotype and were shown to secrete increased levels of inflammatory cytokines including IL-1β and TNFα [25]. We hypothesized that aspirin would reduce the pro-inflammatory status of M1 macrophages and in turn lead to reduced chemerin secretion by adipocytes. To test this, we induced the M1 phenotype in macrophages by LPS stimulation in the absence and presence of aspirin. LPS stimulated macrophages secreted high levels of the inflammatory cytokines TNFα, IL-1β and IL-6. Aspirin co-treatment reduced IL-1β transcription, and IL-1β and IL-6 secretion from macrophages in a dose dependent manner but had no impact on TNFα expression. The higher dose of aspirin (20 mg/l) also significantly increased IL-10 secretion (Figure 2.9). These data show that even low dose aspirin reduces the inflammatory state of macrophages altering the secretion of pro- and anti-inflammatory cytokines. This as a consequence could lead to reduced stimulation of chemerin expression in adipocytes of inflamed adipose tissue.

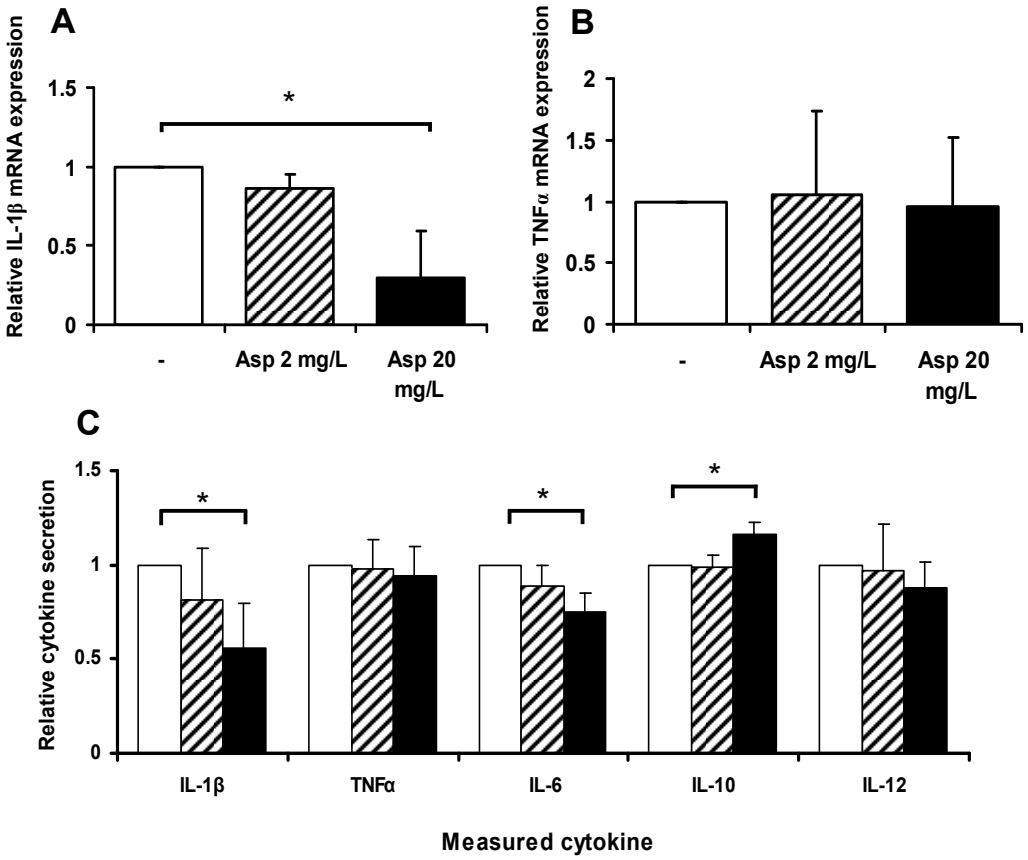

Figure 2.9: Aspirin reduces cytokine expression and secretion of inflammatory M1 macrophages

Human primary macrophages were stimulated for 48 hours with 100 ng/ml LPS for polarization into M1 macrophages in the presence and absence of aspirin. A) IL-1β mRNA in M1 macrophages. B) TNFα mRNA in M1 macrophages. C) Cytokines secreted by M1 macrophages. Macrophages stimulated with LPS alone (white), co-stimulation of LPS with 2 mg/l aspirin (hatched) and with 20 mg/l aspirin (black). mRNA levels were normalized to GAPDH levels and the protein levels were normalized to the control value. The concentration of the secreted cytokines in the supernatant was in the range of pg/ml. Bars represent the mean +/- standard deviation of 3 independent experiments performed in triplicates. * $p < 0.05$, ** $p < 0.01$

2.2.5 Discussion

We show that plasma chemerin levels correlate with multiple risk factors for the metabolic syndrome and CAD. In conformity with previous smaller studies, chemerin levels are positively associated with BMI, total cholesterol, LDL, hsCRP, hypertension, and negatively associated with HDL levels. Despite the association with multiple risk factors for CAD, there is no direct association of chemerin plasma levels with CAD. However, we observed increased plasma chemerin levels in CAD patients not undergoing aspirin treatment, indicating that low dose aspirin reduces chemerin secretion. In scrutinizing the possible mechanism leading to the reduced chemerin secretion, we show that aspirin treatment diminishes the secretion of pro-inflammatory cytokines by M1 activated human primary macrophages possibly leading to reduced stimulation of chemerin transcription and secretion in adipocytes. In this manner, our data generate the hypothesis that low dose aspirin can reduce adipose tissue inflammation by lessening the inflammatory response of macrophages.

Obesity is associated with increased infiltration of macrophages into the adipose tissue [24]. These macrophages have the pro-inflammatory M1 phenotype, while in lean adipose tissue mostly non-phlogistic M2 macrophages are observed [26]. M1 macrophages are a prominent source of inflammatory cytokines, which signal in a parakrine manner to the surrounding cells, and which are also released to the circulation [27]. Consequently, they contribute to the local and to the systemic low-grade inflammation. Our data show that inflammatory cytokines reduce chemerin secretion in hepatocytes and increase chemerin secretion in adipocytes, indicating that inflammation would augment chemerin secretion in the adipose tissue. Considering that hepatocytes in the liver and adipocytes in white adipose tissue are believed to be the major sources of plasma chemerin [23], our findings generate the hypothesis that increased plasma chemerin reflects adipose tissue inflammation. A role for plasma chemerin as a marker for adipose tissue inflammation is supported by the positive correlation of plasma chemerin with the inflammatory cytokines TNFα and IL-6 [28], the negative correlation with adiponectin and HDL [13,29], and the association with various markers of the metabolic syndrome in our and other studies [10,11,12,28]. However, we have to emphasize that the source of chemerin in human plasma has not been fully established and further research is necessary before chemerin can be proposed as a marker for adipose inflammation.

In addition, we show evidence that even low dose aspirin can reduce inflammation and that low dose aspirin treatment is associated with lower plasma chemerin levels in CAD patients. Low dose aspirin is indicated for cardiovascular prophylaxis based on clinical trials showing

that aspirin in all doses reduced the risk of myocardial infarction, ischemic stroke, and vascular death [30]. Aspirin takes effect primarily by inhibiting the thromboxane A_2 (TXA_2) and prostaglandin biosynthesis by cyclooxygenases (COX). Low dose aspirin efficiently inhibits TXA_2 production in platelets, which is necessary for its cardioprotective effects, while higher doses are necessary for the blocking of prostaglandin synthesis - the known anti-inflammatory effect of aspirin [31]. In this context it is noteworthy that platelets store chemerin and release chemerin upon activation by thrombin [32], opening a second pathway for aspirin to reduce plasma chemerin levels through inhibition of platelet activation. However, whether this locally and intermittently released chemerin would substantially contribute to human plasma chemerin levels is questionable and will have to be shown in the future.

Our findings show for the first time that even low dose aspirin has anti-inflammatory effects on primary human macrophages, which may contribute to the clinical benefits of aspirin in the treatment of cardiovascular disease. This is in agreement with the findings of the Physicians Health Study, which showed that low dose aspirin was especially beneficial for the prevention of myocardial infarction in participants with elevated CRP [33]. On the molecular level, there is an indication that that aspirin redirects the COX-2 enzyme from the production of the pro-inflammatory prostaglandins to the production of the pro-resolving aspirin-triggered lipid mediators [34]. These aspirin-triggered lipid mediators induce the resolution of inflammation [35] and are produced especially under low dose aspirin regimens [36]. Intriguingly, aspirin-triggered lipid mediators were also reported to improve the inflammatory status of obese adipose tissue [37,38].

Although plasma chemerin levels were associated with markers of inflammation, and the metabolic syndrome in human studies, it is currently not clear whether increased chemerin levels influence atherosclerosis in humans. Several small case-control studies have been carried out showing contentious results for the association of chemerin plasma levels with CAD. While two small studies in Asians showed an association of plasma chemerin levels with atherosclerotic diseases [39,40], a third study in Caucasians could not corroborate these findings [28]. Our study indicates that we will have to correct for confounding factors such as aspirin treatment to dissect the role of chemerin in human atherosclerosis. In addition, it might be local rather than systemic levels of chemerin that influence atherosclerosis. Systemic overexpression of human chemerin in mice lacking the LDL-receptor did not affect atherogenesis [41]. In contrast, chemerin levels in the epicardial adipose tissue were associated with CAD in Han Chinese [42] and chemerin expression in the periaortic adipose

tissue was shown to correlate with aortic atherosclerosis [17]. These results indicate that locally activated chemerin may influence the local inflammatory state and contribute to atherogenesis, probably through chemoattraction of macrophages [18] and activation of cell adhesion allowing immigration of macrophages into the arterial wall [43].

In conclusion we show that plasma chemerin is associated with systemic markers of inflammation and components of the metabolic syndrome. In addition we show that low dose aspirin treatment reduces pro-inflammatory cytokine secretion by macrophages, which may lead to reduced chemerin secretion by adipocytes and may be a reason for the lower chemerin levels in the circulation of CAD patients on low dose aspirin.

2.2.6 References

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2.2.7 Supplementary data

Supplementary table 2.5: Correlations with CAD risk factors in cases not taking aspirin

	Spearman coef.	**P-value**
BMI	0.24	0.0002
Cholesterol	0.14	0.0224
LDL	0.1	0.1358
hsCRP	0.23	0.0002
HDL	-0.27	0.0000
	T-value	**P-value**
Hypertension	0.24	0.8103

Uni-variate analyses of the association of chemerin with continuous variables were carried out using the Spearman correlation.Two sided t-test was applied to investigate the association of chemerin levels with hypertension.

Supplementary table 2.6: Characteristics of cases with and without aspirin treatment

	Aspirin (n=218)	**No aspirin (n=29)**	**P value**
Age (years)	63 (57;71)*	66 (60; 73)	0.34
Males (%)	78	83	0.77
Body-mass index (BMI)	26.4 (24.4;29.7)	27.9 (25.3;30.3)	0.94
Obesity (BMI ≥ 30, %)	19	28	0.50
Hypertension (%)	47	31	0.15
Diabetes (%)	21	20	1
Current Smokers (%)	17	14	0.92
Ever Smoked (%)	56	76	0.066
Statin treatment (%)	80	83	0.95
Creatinine (μmol/l)	88.0 (79.3;97.0)	91.0 (82.0;108.0)	0.25
hsCRP (mg/l)	1.8 (0.9;3.6)	3.2 (1.8;6.2)	0.15
Cholesterol (mmol/l)	5.2 (4.4-5.9)	4.5 (4.0;6.0)	0.79
HDL (mmol/l)	1.3 (1.1;1.4)	1.1 (1.0; 1.4)	0.029
LDL (mmol/l)	3.3 (2.5-3.9)	2.7 (2.3-3.5)	0.94
Chemerin (μg/l)	168.6 (140.6;195.1)	182.5 (167.3;214.7)	0.009

*median and interquartile ranges are shown. For 2 CAD patients no information about aspirin treatment was available. F-test for the equality of the variances of chemerin levels in the two groups:
F = 1.0407, p-value = 0.8305

Supplementary table 2.7: Primers for quantitative PCR, 5´- 3´

Gene	Forward	Reverse
Human chemerin	TGGAAGAAACCCGAGTGCAAA	CCCCATAGAGACCCAAGTTCT
Human GAPDH	CCCATGTTCGTCATGGGTGT	TGGTCATGAGTCCTTCCACGATA
Mouse chemerin	TACAGGTGGCTCTGGAGGAGTTC	CTTCTCCCGTTTGGTTTGATTG
Mouse cyclophilin A	GAGGTGTTTGCAGACAAAGTTC	CCCTGGCACATGAATCCTGG
Human IL-1β	TACCTGTCCTGCGTGTTGAA	TCTTTGGGTAATTTTTGGGATCT
Human TNFα	GAGTGACAAGCCTGTAGCCC ATGTTGTAGCA	GGCAATGATGATCCCAAAGTAGAC CTGCCCAGACT

2.3 Single nucleotide polymorphisms in the ChemR23 gene and susceptibility to coronary artery disease

Unpublished data

2.3.1 Introduction

A leading cause of mortality worldwide, coronary artery disease (CAD) is caused by plaque formation in the intima of mid-sized and large blood vessels – a process termed atherosclerosis. Atherosclerotic plaques are marked with lipid retention and chronic inflammation in the plaque [95], and it has been suggested that the progression of the atherosclerotic plaque development is propelled by impaired resolution of inflammation within the plaque [102].

Macrophages are important players in the pathology of the atherosclerotic plaque. They infiltrate the vascular intima taking up oxidized LDL-cholesterol particles during plaque formation initiation [90]. Lipid laden macrophages, so called foam cells, and activated macrophages then fill the center of the plaque. They secrete a spectrum of inflammatory cytokines and chemokines, which activate other cells within the plaque and attract further immune cells from the circulation, increasing the inflammatory milieu in the plaque [182]. On the other hand, macrophages play a key role in the resolution of inflammation and can have a beneficial function in atherosclerosis. Alternatively activated macrophages (M2 macrophages) clear apoptotic cells by phagocytosis preventing necrosis within the plaque [183], and synthesize matrix repair proteins that stabilize vulnerable plaques [184]. In addition, the anti-inflammatory cytokines IL-10 and TGFβ typically secreted by M2 macrophages have been shown to be atheroprotective [100,185].

ChemR23 is a G-protein coupled receptor expressed on monocytes and macrophages [128]. It binds two ligands – the peptide chemerin and the lipid resolvin E1 (RvE1) [47,135]. Chemerin is secreted in inflamed tissue and serves as a chemoattractant of ChemR23 expressing cells contributing to the initiation of inflammation [136]. Chemerin has been detected in atherosclerotic plaques and epicardial adipose tissue [186,187], and both chemerin and its receptor ChemR23 are expressed on foam cells [188]. RvE1 is a metabolite of the omega-3 polyunsaturated fatty acid (PUFA) eicosapentaenoic acid and has proresolving properties [33]. It induces macrophage phagocytosis of pathogens and apoptotic neutrophils [48]. In addition, RvE1 likely one of the mediators of beneficial effects of dietary PUFA [31], which have been shown to have cardio-protective role [189].

The above mentioned findings lead us to the assumption that as a receptor of chemerin and RvE1, ChemR23 plays an important role in the course and resolution of inflammation within atherosclerotic plaques. We therefore hypothesized that functional polymorphisms in the ChemR23 gene will alter these processes and thus may have an impact on the onset and/or course of atherosclerosis. Here, we screened both promoters and the single coding exon of the ChemR23 gene for polymorphisms, and investigated their association with CAD in a case-control study.

2.3.2 Material and Methods

Case control study

A total of 328 men and 149 women from Zurich volunteered to participate in the study. They all signed an informed consent form. The study was approved by the local Ethics Committee and complies with the Declaration of Helsinki. The case group consisted of 249 consecutive Caucasian patients with angiographically documented CAD with more than 50% stenosis in at least 1 coronary artery. The control group consisted of 228 Caucasians with angiographically negative results and individuals with no history of CAD, stroke, or peripheral vascular disease recruited from the general population. Risk factors and the use of medication were assessed by a questionnaire. Clinical chemistry analyses were carried out on a Roche-Hitachi Modular Clinical Chemistry analyzer using commercial tests from Roche Diagnostics (Rotkreuz, Switzerland). The case-control study has a power of 25% - 80% to detect an odds ratio (OR) of 1.7 for minor allele frequencies of 5-30%.

Sequencing

All three analyzed regions were sequenced on the 3130xl Genetic Analyzer (Applied Biosystems). After amplification of the fragments by PCR, sequencing reaction was performed with 3 µl purified PCR products in 10 µl reaction volume with the BigDye Terminator v 1.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. The primers used are listed in Table 2.3..

Table 2.3: Primers for PCR and sequencing, 5´- 3´

Region	Forward primer	Reverse primer
Promoter 1	TTAACAGGCAAGGACAGCACT	CCAAGGCAAGTGTGTGATGA
Promoter 3	TTGGGGCCACCAAAGGCACC	CCCACCTCCCTATGTTAGTTC
Coding region	CTGGCATGTAGGAGGTACCAGAG	TGGCCTGTATCTTCACTTAGAGC

Statistical analysis

The statistical analysis was performed using the R software 2.12.0. The association of SNPs with CAD were analyzed by binary logistic regression in an additive genetic model adjusted for sex and age. P-values lower than 0.05 were considered statistically significant. The haplotype and linkage analysis were performed with Haploview 4.2.

2.3.3 Results

To investigate whether polymorphisms in the ChemR23 gene are associated with atherosclerosis, we screened in 100 individuals the coding region, the non-coding exon 1, and the promoters P1 (chromosome 12, 12q24.1, upstream of 108733094) and P3 (upstream of 108711724), which we have previously characterized as active in monocytes and macrophages. Table 2.4 summarizes the detected polymorphisms and their allele frequencies.

Table 2.4: Summary of found SNPs

Location	SNP	Minor allele frequency (%)
Promoter 1	T>C rs73403360	0.4% (private mutation)
Exon 1	c.- 192 - 195 del AGAA	0.4% (private mutation)
	c.- 161 del C	0.7%
Promoter 3	G>A rs1399821	2.9%
	T>C rs1399820	67.8%
	G>A rs11113814	2.9%
	T>C rs184437753	0.1% (Private mutation)
	A>G rs10735428	68.0%
Coding region	T>G rs141421422	0.3%
	T>C rs192034694	0.8%
	C>T rs61740671	2.3%
	C>T rs146650378	0.1% (private mutation)
	G>C rs1057401	43.7%

Subsequently, we sequenced the coding region and the promoter region P3, where single nucleotide polymorphisms (SNPs) with sufficient frequencies were found to be analyzed for association with CAD in our case-control study. In the promoter P3, two pairs of variations were shown to be inherited together: rs1399821 and rs11113814 were 100% linked in our sample, and rs1399820 and rs10735428 were linked in 98%. The C allele of rs1399820 was significantly associated with CAD (OR = 1.36; confidence interval (CI) = 1.02 – 1.81; p-value: 0.034), and there was a trend for association for the G allele of rs10735428 with CAD (OR = 1.27; CI = 0.96 – 1. 69; p-value: 0.094) (Table 2.5A). However, both rs1399820 and rs10735428 were not in Hardy-Weinberg equilibrium in controls, which showed higher numbers of the homozygote of the minor allele. (In a dominant model, the C and G are more strongly associated with atherosclerosis). None of the variations changes any predicted transcription factor binding site as analyzed using MatInspector from Genomatix [190]. The rs1399820 is predicted to create a new binding site for the SAM pointed domain containing transcription factor from the ETS family.

In the coding region, no significant association between the detected polymorphisms and CAD was observed. However, the non-synonymous polymorphism rs192034694 showed a trend to increase the risk for CAD (OR = 3.8; CI = 0.79 – 27.60; p-value: 0.12) (Table 2.5B). In addition, this polymorphism causes the change of isoleucine to threonine at the position 64, which is located in the predicted 1st transmembrane domain (source: UniProt [191]) and is rated as damaging by the software PolyPhen-2 [192]. The non-synonymous polymorphism rs1399821 leads to the exchange of isoleucine to serine in the same domain and was also rated damaging by PolyPhen-2. However, it's frequency is too low to establish a reliable association with CAD in the presented case-control study.

Table 2.5: Logistic regression models predicting case status

A. Promoter P3

	Allele frequency (%)	Genotype Controls	Genotype Cases	Odds ratio	97.5%CI	p-value
G>A rs1399821	G: 97.1 A: 2.9	223 / 10 / 0	238 / 16 / 1	1.91	0.86 – 4.57	0.123
T>C rs1399820	T: 32.2 C: 67.8	34 / 91 / 103	17 / 113 / 119	1.36	1.02 – 1.81	0.034
G>A rs11113814	G: 97.1 A: 2.9	223 / 10 / 0	238 / 16 / 1	1.91	0.86 – 4.57	0.123
A>G rs10735428	A: 32.0 G: 68.0	33 / 89 / 106	19 / 111 / 119	1.27	0.96 – 1. 69	0.094

B. Coding region

T>G rs141421422	T: 99.8 G: 0.2	227/ 1 / 0	248 / 1 / 0	0.96	0.04 – 24.80	0.97
T>C rs192034694	T: 99.2 C: 0.8	226/ 2/ 0	243 / 6 / 0	3.80	0.79 – 27.60	0.12
C>T rs61740671	C: 97.7 T: 2.3	215 / 13 / 0	240 / 9 / 0	0.50	0.20 – 1.24	0.14
G>C rs1057401	G: 56.1 C: 43.9	70 /120 / 38	80 / 115/ 54	1.08	0.82 – 1.41	0.58

Logistic regression using an additive genetic model adjusted for age and sex.
P values < 0.05 are considered significant.

Haplotype analysis of all detected SNPs has shown a high recombination frequency between the promoter P3 and the coding region. We observed 6 haplotypes with a frequency >1%. The haplotype frequencies are outlined in Table 2.6. None of the haplotypes was associated with CAD.

Table 2.6: Observed haplotypes

	Haplotype 1	Haplotype 2	Haplotype 3	Haplotype 4	Haplotype 5	Haplotype 6
rs1399821	G	G	A	A	G	G
rs1399820	G	G	G	G	A	A
rs11113814	C	C	T	T	C	C
rs10735428	G	G	G	G	A	A
rs141421422	T	T	T	T	T	T
rs192034694	T	T	T	T	T	T
rs61740671	C	C	C	C	C	C
rs1057401	G	C	G	C	C	C
Frequency	**0.56**	**0.26**	**0.16**	**0.14**	**0.02**	**0.02**

2.3.4 Discussion

We have scanned the coding region, exon 1 and two promoters of the ChemR23 gene for polymorphisms, and analyzed their association with atherosclerosis in a CAD case-control study. The polymorphism rs1399820 was significantly associated with CAD in our case control study. However, as the odds ratio is relatively low, we assume that this association might be due to chance rather than a real association, although *in-silico* analysis showed that rs1399820 might create a new binding site for the SAM pointed domain ETS factor (SPDEF). SPDEF belongs to the Ets family of transcription factors that regulate many developmental processes including cell lineage specification, proliferation, differentiation, angiogenesis, and apoptosis [193]. Impaired activation and expression of SPDEF has been associated with prostate and breast cancer [194,195]. SPDEF is highly expressed in epithelial cells of lung, prostate, intestine and mammary glands [196] but it is unclear whether it is expressed by mononuclear cells. Therefore this possible creation of a new binding site might not be relevant in monocytes and macrophages. A transcription activity assay will have to be done to establish, whether this SNP changes the activity of the promoter P3 in macrophages.

In the coding region, two relatively rare SNPs were detected (rs192034694 and rs141421422), causing a change of an amino acid in the predicted 1st transmembrane domain. Both of these changes were classified as deleterious in a *in silico* analysis using the software PolyPhen-2. In addition, rs192034694 showed a trend to increase the risk for CAD. A larger study would be necessary to confirm the association of this polymorphism with CAD. In addition, it would be desirable to analyze the impact of both rs192034694 and rs141421422 on the functionality of the receptor. This could be done by performing chemerin chemotaxis assay with macrophages from carriers of these SNPs or measuring chemerin triggered calcium release in SNP bearing ChemR23 transfected cells [133].

In conclusion, the promoter variation rs1399820 showed an association while the the non-synonymouse coding region variation rs192034694 showed a trend for association with CAD. The variations may have an impact on the regulation and functionality of ChemR23, respectively. Further *in vitro* studies would be necessary to confirm the predicted effects. In addition, a larger case-control study will be necessary to confirm their association with CAD.

3 Discussion

ChemR23 expression and function in differentially activated macrophages

A receptor of the lipid mediator resolvin E1, and of the chemoattractant and adipokine chemerin, ChemR23 has a role in the resolution of inflammation, chemotaxis of mononuclear cells to the inflamed area, and in adipogenesis. In addition, the ChemR23 - chemerin axis has been proposed to present a link between obesity and excessive accumulation of inflammatory leukocytes in the adipose tissue [146]. Yet, there is little known about the regulation of ChemR23 expression during the course of inflammation or in response to inflammatory and anti-inflammatory stimuli.

We show that ChemR23 expression increases during monocyte differentiation to macrophages and is further induced by inflammatory stimuli. Naive and inflammatory M1 macrophages are thus chemotactic toward chemerin and respond to RvE1 signaling by the increase of IL-10 transcription. In contrast, anti-inflammatory M2 macrophages do not express ChemR23 and are not responsive to chemerin.

Macrophages are after adipocytes one of the most abundant cell types in the adipose tissue. Increased infiltration of monocytes/macrophages into the adipose tissue is associated with obesity [197,198]. In addition, these macrophages have mostly the M1 phenotype in contrast to M2 macrophages, which populate the lean adipose [79,199]. Chemerin is highly secreted by mature adipocytes and chemerin secretion from the adipose tissue has been shown to increase with increasing body weight [172]. This adipocyte secreted and activated chemerin [138] is likely to contribute to the inflammatory status of obese adipose tissue by attraction of macrophages. Our results support this notion showing that only naive and inflammatory but not M2 macrophages express ChemR23 and thus can migrate toward chemerin. M1 macrophages secrete increased amounts of inflammatory cytokines such as TNFα and IL-1β, which signal to adipocytes in a parakrine manner. We show that these inflammatory cytokines increase chemerin transcription and secretion in adipocytes possibly creating a vicious circle. On the other hand, RvE1 in our experiments increases IL-10 transcription in M1 macrophages leading to an intermediate phenotype and revealing a possible role of RvE1 in the resolution of adipose tissue inflammation.

Anti-inflammatory actions of low dose aspirin

Coronary artery disease is tightly associated with inflammation manifesting itself by increased CRP and IL-6, and reduced IL-10 levels in plasma [101,200,201,202]. We show that chemerin plasma levels in CAD patients not treated with aspirin are significantly increased in

comparison to patients on low dose aspirin and to healthy controls. *In vitro*, low dose aspirin treatment decreased the secretion of IL-1β and IL-6 and increased the anti-inflammatory IL-10 in M1 activated macrophages but had no direct effect on chemerin expression in the major chemerin secretors hepatocytes and adipocytes [124,143]. Chemerin secretion is upregulated by IL-1β and TNFα in adipocytes but mildly reduced in hepatocytes. This indicates that inflammation will increase chemerin secretion from adipocytes while the anti-inflammatory effect of low dose aspirin on macrophages may result in reduced systemic and adipose tissue inflammation leading to reduced chemerin secretion. In line with our finding, reduced IL-1β plasma levels have been observed after 2 months of low dose aspirin treatment in patients with hypercholesterolemia [203]. To confirm that low dose aspirin reduces systemic and adipose tissue inflammation it would however be important to assess whether it reduces IL-1β and IL-6 and increases IL-10 in plasma and/or within the adipose tissue. Unfortunately, we were not able to do these measurements in our CAD case-control study. Nevertheless, we observed a trend in CRP reduction in low dose aspirin treated patients. However, as the group not on aspirin was relatively small the two groups were too unequal for detecting a statistically significant difference. Further research will be necessary to confirm that low dose aspirin treatment reduces adipose inflammation and so chemerin secretion from adipose tissue *in vivo*. It would be expecially interesting to analyze whether low dose aspirin treatment affects plasma concentrations of chemerin and inflammatory markers in obese patients and whether adipose tissue explants secrete lower levels of chemerin and/or inflammatory cytokines in response to aspirin treatment.

We show that aspirin down-regulates IL-1β on the transcription level while it has no effect on TNFα transcription and secretion. Classically, secretion of inflammatory cytokines including TNFα and IL-1β is induced in macrophages after LPS binding to the TLR-4 receptor through activation of the MyD88-dependent signaling pathways. This leads to NF-κB translocation to the nucleus and to initiation of transcription [204,205]. Previously, aspirin has been reported to block NF-κB signaling [206] and inhibit TNFα expression in mouse primary macrophages [207]. Therefore, it could be expected that aspirin treatment reduces both TNFα and IL-1β. However, the aspirin concentrations used in all previous experiments were 100 – 1000 times higher that the concentrations corresponding to low dose of aspirin treatment used in our experiment. The fact that low dose aspirin reduces IL-1β but not TNFα transcription in our experiments, indicates that low dose aspirin acts in a NF-κB independent manner. Such a pathway was recently described in a study showig that LPS-stimulated IL-1β transcription is

specifically regulated by the hypoxia induced factor HIF-1α. The inhibition of HIF-1α stabilization with succinate suppressed LPS-induced IL-1β but not TNF-α [208].

We further report that aspirin treatment increases IL-10 secretion in M1 macrophages. IL-10 was previously shown to have anti-inflammatory effects on mouse 3T3 adipocytes suppressing the production of CCL2 and antagonizing the effect of TNFα in a NF-kB independent manner in these cells [80]. The aspirin stimulated increase in secretion of IL-10 in M1 macrophages obeserved in our experiments might thus contribute to reduction of chemerin secretion in adipose tissue. The effect of IL-10 on human adipocytes has to be however further studied because the IL-10 receptor was shown to be transcribed in human adipocytes [73] but its presence on the surface of human adipocytes has been questioned [73,80,209].

Finally, aspirin treatment of M1 macrophages also lead to reduction of IL-6 secretion. IL-6 is a pleyotropic cytokine increased in plasma during obesity and systemic inflammation [210], and increased IL-6 levels in plasma have been associated with increased risk for myocardial infarction [211]. Although IL-6 does not directly affect chemerin transcription and secretion in adipocytes, the decrease of its secretion by M1 macrophages can serve as an additional indication that low dose aspirin indeed reduces the inflammatory status of these macrophages. In line with our finding, low dose aspirin treatment was shown to reduce IL-6 levels in obese individuals and the IL-6 secretion by white adipose tissue [212] further supporting the notion that low dose aspirin might reduce systemic and adipose tissue inflammation.

Aspirin is a multifunctional drug and has, next to the reported anti-inflammatory effects on macrophages, systemic effects on different cell types and tissues in the body. Aspirin triggered resolvins and lipoxins secreted by different tissues after the intake of aspirin are likely additional explanation for the anti-inflammatory effect of low dose aspirin [213,214]. Resolvins and aspirin-triggered resolvins were in addition shown to have a beneficial effect on inflammation associated with obesity. Resolvins of the D-type have been reported to improve the inflammatory status of obese adipose tissue by increasing adiponectin secretion and decreasing TNFα, IL-6 and IL-1β secretion in mouse adipose tissue explants [63,215]. In addition, stimulation of LPS activated mouse M1 macrophages with RvD1 decreased TNFα and IL-6 secretion and increased arginase 1 expression (mouse M2 macrophage marker) in a way similar to IL-4 re-stimulation [215]. Although the receptor of RvE1 is expressed on both adipocytes and M1 macrophages, its direct effects in adipose tissue have not been studied. However, intraperitoneal injection of RvE1 had an insulin sensitizing effect on the adipose tissue by inducing adiponectin, glucose receptor (GLUT-4), insulin receptor substrate (IRS-1)

and PPARγ expression in *ob/ob* mice [64]. Whether this effect was due to RvE1 signaling on adipocytes or in macrophages is not clear. We show that RvE1 increased anti-inflammatory IL-10 transcription in human M1 macrophages. This was in contrast to IL-4 signaling in human M1 macrophages which had no impact on IL-10 transcription but lead to increased CD206 expression. This indicates that RvE1 signals in a different way than IL-4 and does not lead to M2 phenotype in macrophages but rather to an intermediate phenotype. Such macrophage phenotype has been described during the resolution phase of zymosan induced murine peritonitis [5]. Further experiments have to follow to show whether RvE1 has anti-inflammatory or proresolving effects on adipocytes, and macrophages within the adipose tissue by analyzing the impact of RvE1 on adipocytes and adipose tissue explants in culture.

Use of alternative promoters and alternative splicing of the ChemR23 mRNA in macrophages

Up to now the promoter region of ChemR23 has not been characterized in humans. We have identified two promoters (P1 and P3) active in human monocytes and macrophages, and 1 additional putative promoter (P2) with low activity in macrophages. A recent large scale computational analysis of 5´end sequences of putative full length cDNAs revealed that more than 50% of human genes are transcribed from alternative promoters [216]. Similar to ChemR23, in many genes where alternative promoter usage has been experimentally confirmed, no variation in the translated protein has been reported [217]. Instead, the use of alternative promoters is a means of diversification of transcription regulation in different cell types, different developmental stages or in response to different stimuli [218]. In the case of ChemR23, the identified putative promoter P2 with low activity in macrophages may allow differential regulation of transcription of ChemR23 in adipocytes or endothelial cells, where ChemR23 has been shown to be also highly expressed [125,143]. In monocytes and macrophages, ChemR23 alternative promoter usage leads to transcription of 2 alternative first exons. Additionally, mRNAs transcribed from promoter P1 undergo alternative splicing leading to 2 mRNAs variants in macrophages and 3 in monocytes. This alternative splicing of the 5´untranslated region (UTR) of mRNAs enables tight regulation of mRNA translation through different stability or variable translation efficiency of individual isoforms [218]. We have previously shown that the 5´UTR of the LXA_4 receptor (FPR2) is differentially spliced leading to mRNA isoforms with different translation efficiencies in human monocytes and macrophages [219]. It is likely that the alternative splicing of the non-coding exons in ChemR23 mRNA has a similar function. A luciferase activity assay with different 5´UTR fragments subcloned between the SV40 promoter and the luciferase gene could be done to analyze the translation efficiency of individual mRNA isoforms.

Conclusion

To conclude, we show that ChemR23 expression is tightly regulated in monocytes and macrophages. ChemR23 expression is upregulated during monocyte differentiation to macrophages and further amplified by inflammatory stimuli. In contrast, ChemR23 is not present on the membrane of M2 macrophages. Our data support ChemR23 function in chemotaxis of monocytes, and naive and inflammatory M1 macrophages. On the other hand, M2 macrophages, which have anti-inflammatory properties and are associated with wound healing and tissue repair [13] were not responsive to ChemR23 mediated stimuli in our experiments. We therefore surmise that the proresolving actions of RvE1 are mediated through ChemR23 in naive or inflammatory M1 macrophages. A direct re-polarization of M1 to M2-like macrophage phenotype stimulated by cytokines was documented *in vitro* [19,220]. A switch of macrophage phenotypes has been also observed during transition of acute to resolving inflammation *in vivo* [14]. We show that RvE1 increases IL-10 transcription in M1 macrophages shifting them toward an anti-inflammatory phenotype. However, IL-10 secretion is not increased after 4 days of RvE1 treatment. It is possible that RvE1 acts more rapidly and IL-10 is increased earlier after RvE1 application. A time-course measurements of IL-10 in the macrophage media could be done to show whether this is the case. On the other hand, RvE1 might act only as a subtle stimulus on macrophages and a more complex tissue context might be needed to achieve a complete re-polarization for example in response to other signals triggered by RvE1 through ChemR23 on endothelial cells [125] and through BLT1 on neutrophils [49]. In addition, it has been shown that macrophage efferocytosis (phagocytosis of dead cells and of cellular debris) induced the switch of macrophage phenotype toward a pro-resolving one, and increased IL-10 secretion [221,222,223]. RvE1 accelerated this switch of macrophage phenotype by reducing the efferocytic requirement for it [222]. Therefore a combination of efferocytosis and RvE1 triggering might be neccessary for a full re-polarization of macrophages to a pro-resolving phenotype.

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Own contribution to publications

Low Dose Aspirin is Associated with Plasma Chemerin Levels and May Reduce Adipose Tissue Inflammation

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ELISA chemerin measurements, statistical analysis, cell culture experiments, manucript writing

ChemR23, Receptor of Chemerin and Resolvin E1 is expressed and functional on M1 but not on M2 macrophages

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5´RACE, luciferase activity experiments, RT-PCR, cell culture experiments except FACS measurements and chemotaxis assay, data evaluation, manuscript writing

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Curriculum Vitae

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Publications

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2. Waechter, V., Schmid, M., Herova, M., Weber, A., Gunther, V., Marti-Jaun, J., Wuest, S., Rosinger, M., Gemperle, C., and Hersberger, M. *J Immunol* 188(4), 1856-1867

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Prizes

2010 - poster prize at the Symposium of the Zurich Center for Integrative Human Physiology